STUDIES IN EXPERIMENTAL ATHEROSCLEROSIS.

A Preliminary Report.*

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Plates 7 to 10.

In the present communication the protocols of the experiments will not be reproduced in detail, nor will the extensive literature that has gathered about the problems of atherosclerosis be discussed; and lastly, definite conclusions as to the result of the experiments will not be warranted. It is reserved for a future communication, when experiments still under way will have been concluded, and more definite knowledge as to the histological and chemical results may have been obtained, to report more in detail and with a critical appreciation of the literature.

All the investigations here reported have been done on dogs. The spontaneous occurrence of atherosclerosis in dogs, its histological structure, and the question of its greater or less analogy to human atherosclerosis are problems that have not received the attention that they deserve. In the text-books on animal pathology, little is to be found on the subject. Until recently the general opinion appears to have been that dogs were not disposed to atherosclerosis, and that if it occurred at all it was exceptional. Latterly it has been discovered that dogs are indeed subject to spontaneous atherosclerosis. The process is said to be limited, however, to the large vessels. The early stages appear as round or more oval or lengthwise patches of a whitish or pale yellowish and pinkish color, and are somewhat elevated above the surface of the intima. These sclerotic areas, it is said, are met with principally in localizations where the blood is apt to impinge with greater force against the wall of the vessel; for example, places where the vessel takes a sudden

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turn in a new direction, or where other vessels branch off from the main trunk. The later stage of the atherosclerotic process is described in text-books as showing atrophy of intima and media, extensive fatty degeneration and calcification.[1] Della Vida[2] has seen spontaneous atherosclerosis in a large percentage of old dogs.[3]

Notwithstanding, however, that up to the present our knowledge of atherosclerotic processes in dogs is scant and insufficient, there seems to be no reasonable doubt that a process in many respects analogous to human atherosclerosis does occur. Granting this, for many years the writer has believed, and quite independently of experiments on dogs published in recent years, that if the possibility existed of producing experimentally a process that resembled in any way human atherosclerosis, one should be able to produce it in dogs, and not alone in rabbits. As the dog is omnivorous, it might be assumed, although it does not necessarily follow, that the metabolic conditions would approach more nearly those of human beings than is the case in the herbivorous rabbit. It is also an advantage that the disturbing median necrosis and calcification to which the aorta of the rabbit is readily disposed, is negligible in experiments on dogs. It is as yet not possible to state with certitude whether the structure of the aorta of dogs resembles the human aorta more closely than does that of the rabbit.

Much has been said and written concerning high blood pressure; various intoxications, such as lead and nicotin, and also infections with bacteria and bacterial products are important factors in the etiology of atherosclerosis. It was proposed to investigate some of these questions experimentally.

The first series of experiments was done during the greater part of 1908 and the first half of 1909.[4]

In this entire series the material to be tested was applied parenter-

[1] Hutyra, F., and Marek, J., Specielle Pathologie und Therapie der Haustiere, 3d edition, Jena, 1910, ii, 1100. Lyding, H., Zur Kenntnis der Arteriosklerose bei Haustieren, *Ztschr. f. Thiermed.*, 1907, xi, 359.

[2] Della Vida, M. L., Ueber experimentelle Arteriosklerose, *Deutsch. med. Wchnschr.*, 1913, xxxix, 2200.

[3] Celli, A., Manuale dell' igienista, 4th edition, Turin, 1912, ii, pt. 2, 1148.

[4] The writer wishes to express his thanks to Dr. William H. Park, through whose courtesy and interest it was possible to carry on this work in the laboratories of the New York Department of Health.

ally. The injections were supposed to be made into the jugular vein, with all proper regard to asepsis. It is not always easy to get the needle into the jugular vein, and it probably happened very often that the injection became subcutaneous instead of intravenous.

LEAD.

Former experience with rabbits has shown that the ordinary soluble lead salts were unsuited for hypodermic application that was to extend over any considerable length of time. The local reactions in every case were such as soon to render the injections impossible. It was found, however, that the triethylacetate of lead, as employed by Harnack[5] in his experiments, served our purpose admirably. Dr. Robert Emerson, then of Boston, prepared the salt in perfect purity. There was little or no local reaction, and the injections could thus be carried on *ad libitum*.

It should be mentioned that the blood pressure of our dogs was not tested, but that Harnack, contrary to the generally accepted opinion, found that in his animals the lead injections caused the blood pressure to rise. Two dogs were tested.

Dog 1.—The injections were commenced on Apr. 3, 1908. The animal was found dead in his cage on May 26, 1908. The symptoms corresponded closely to those described by Harnack. The temperature ranged from 101° to 104 3/5° F. There was considerable excitement, diarrhea, towards the end of the experiment bloody stools, and paralysis of hind legs. During the 55 days of the experiment the dog received injections amounting to 290 minims of a 1 per cent. solution.

Dog 2.—From June 1, 1908, to Aug. 18, 1908, when the dog was found dead. The symptoms were the same as in the first dog. The total amount injected was 590 minims. In both dogs no lesion could be found in the aorta or any other large vessel.[6]

NICOTIN.

Dog 1.—The experiment lasted from May 4 to Dec. 6, 1908, a period of about 7 months. The dog was found dead in the cage. 15 minims of a 1 per cent. solution of nicotin were injected at varying intervals, according to the condition of the dog, mostly every 2 or 3 days. There were never any convulsions. No lesions were present in the arteries.

[5] Harnack, E., Über die Wirkung des Bleis auf den thierischen Organismus, *Arch. f. exper. Path. u. Pharmakol.*, 1878, ix, 152.

[6] Harnack also failed to find any lesion in the vascular system of his dogs.

DIPHTHERIA TOXIN.[7]

Dog 1.—From May 12 to July 18, 1908,—about 67 days. The solution used was 1:100. Commencing with a single drop, the quantity injected was gradually increased until 1 c.c. was injected, after which the dog was found dead in the cage. No sclerotic lesions were present in any of the vessels.

Dog 2.—From Apr. 4 to Apr. 26, 1908. Death after 22 days. Temperature up to 104 1/5° F. One drop of the toxin was injected; twice only 2 drops. The result as to the arteries was negative.

Dog 3.—From Mar. 17 to Apr. 9, 1909. The solution used was 1:150. 1 to 10 drops were injected. Death in about 3 weeks. Temperature up to 104 1/4° F. Negative result.

EXPERIMENTS WITH BACTERIA.[8]

For the success of these experiments it was necessary to keep the animals alive as long as possible, but in a condition of mild sepsis. For this reason cultures of but moderate virulence were employed.

STAPHYLOCOCCUS AUREUS.

Dog 1.—Experiments commenced Mar. 10, 1908, and the dog was killed on June 13, 1908, after about 3 months. The injections, commencing with a single loop, suspended in normal saline, were gradually increased to the injection of an entire agar slant. The temperatures ranged from 100° to 104 2/5° F., showing the characteristic septic temperature curve. Result as to blood vessels negative.

Dog 2.—Oct. 30, 1908, to Mar. 15, 1909. Animal found dead after about 4 1/2 months. In this case the cultures were not so virulent as in the former experiment, and the sepsis was therefore not so marked, though nearly always an entire slant was injected. Temperature from 101° to 103 4/5° F. No arterial lesion.

Saltykow reported some time ago that he had obtained positive results from the injection of staphylococcus in rabbits. In his most recent publication,[9] however, he is inclined to attribute the positive result not to the injections of staphylococcus, but to the milk with which the rabbits were fed.

STREPTOCOCCUS PYOGENES ALBUS.

Dog 1.—From Oct. 30, 1908, to death, a little over 5 months, on Apr. 9, 1909. Cultures were not very virulent. Whole agar slants were used. Temperature varied in the neighborhood of 103° F. Result as to arteries negative.

[7] The diphtheria toxin was procured from the laboratories of the New York Department of Health.

[8] The cultures were obtained from the Board of Health laboratories, and were of various strains. I am also indebted to Dr. Alfred F. Hess, who controlled the purity of the cultures.

[9] Saltykow, S., Experimentelle Atherosklerose, *Beitr. z. path. Anat. u. z. allg. Path.*, 1914, lvii, 415.

COLON BACILLUS.

Dog 1.—From Mar. 10, 1908, to Oct. 15, 1908. Animal killed after about 7 months. Temperature ranged between 99 3/5° and 103 2/5° F. Animal gained weight during the experiment. Arteries normal.

Dog 2.—Oct. 30, 1908. Found dead after 5¾ months, on Apr. 17, 1909. The animal had developed a number of abscesses. Temperature ranged from 102° to 104° F. Arteries normal.

TYPHOID BACILLUS.

Dog 1.—Oct. 30, 1908. Killed after about 6 months, on Apr. 30, 1909. Temperature ranged from 101 1/2° to 103 1/5° F. Profuse diarrhea and frequent bloody stools. Arteries without lesion.

I was compelled to interrupt these studies for a number of years, but was enabled to take them up again in January, 1913.[10]

It has been shown in former experiments that the injection of adrenalin alone seemed to have no effect upon the arteries of the dog.[11] It was now proposed to test the effect of the high blood pressure caused by the adrenalin, combined with severe bodily exertion, with special reference to the purely mechanical *"Abnutzungstheorie"* that had at one time been generally and favorably received. For this purpose, a treadmill was constructed, so that two dogs could run on it simultaneously and at the same speed, and could not stand still during the period of exercise; they were, moreover, so harnessed that they could not lie down. One dog was injected with gradually increasing doses of adrenalin 1 to 1,000, usually every other day, sometimes several days in succession. It was intended to inject the adrenalin into the jugular vein, but the injections were probably mostly subcutaneous.[12]

The second dog served as a control; it received no injection, but worked steadily together with the other dog in the treadmill. The

[10] I wish to express here my grateful obligation to Professor Frederic S. Lee, Director of the Physiological Department of the College of Physicians and Surgeons of Columbia University, for placing the conveniences of his laboratory at my disposal.

[11] Otto, C., Über Arteriosklerose bei Tieren und ihr Verhältnis zur menschlichen Arteriosklerose, *Virchows Arch. f. path. Anat.*, 1911, cciii, 352. Otto finds that dogs are much less sensitive to adrenalin than rabbits, but claims some positive results in the dogs' aortas after the injection of astoundingly large quantities of adrenalin.

[12] I am indebted to Dr. B. S. Oppenheimer for carrying out the injections and for performing the autopsies on both dogs.

experiment was started on January 20, 1913. Running in the treadmill began with twenty-five minutes in the morning and thirty minutes in the afternoon. Occasionally for a day or two there was no running, but on the whole the work went on fairly steadily, and was increased gradually so that at the end of the experiment, on June 2, 1913, when both dogs were killed, they ran at top speed for almost four hours in the morning and about three hours in the afternoon. The adrenalin injections began with ten minims, and the dose was gradually increased so that at the end of about four months the dog received eighty minims at a single injection, the total amount injected in sixty injections during 130 days being 1.676 grams. These doses are minute compared to the huge quantities injected by Otto,[13] who injected 49.5 grams of pure adrenalin in the course of five months in seventy-eight injections. The initial weight of the adrenalin dog was 11,870 grams; he gradually gained in weight, so that on April 4 he reached his highest weight of 15,300 grams; from then on he gradually lost again, so that at death he had again come down to about his original weight. The control dog weighed 10,220 grams, on March 16 had gained about 2 kilos, and at death weighed about 8,780 grams. A very careful study of the blood vessels after death failed to show any arterial lesion in either dog.

It was soon noticed that, while the adrenalin dog was active and happy, and was practically untiring in running the treadmill, and always at top speed, the control dog was very soon tired, dispirited, and at the end of his forced run completely played out. It is worth noting, also, that the adrenalin dog developed no glycosuria. These facts correspond with Cannon's recently expressed views.

The uniformly negative result of all these experiments with reference to the arteries was calculated to impress one very strongly with the conviction that mere mechanical conditions, as well as intoxications and bacterial infections of various kinds, were not, at least in the dog, determining factors, and that parenteral methods were not likely to lead to any results. In the meantime, moreover, the researches of Aschoff, Anitschkow, Chalatow, Ignatowsky, Saltykow, Steinbiss, and others had been published, from which it appeared that lesions resembling human atherosclerosis very closely

[13] Otto, C., *loc. cit.*

could be produced principally in rabbits, by feeding these animals on proteins and lipoids, especially cholesterin. Following along these lines, and on the theory that atherosclerosis was probably caused by chemical rather than by mere mechanical influences, another set of experiments was undertaken to test the possible effects of various kinds of chemical substances added to the food of the animals.

From the fact that chondroitin sulphuric acid can be obtained in comparatively large quantities from the arteries, and especially the aorta, it was thought that adding this substance to the ordinary food of dogs might possibly give some results.[14]

The dog, a healthy male fox-terrier, was fed in the usual way with bread and meat. On Oct. 24, 1913, a capsule containing 0.5 gm. of the soda salt of chondroitin sulphuric acid was added to his daily meal until Nov. 25, 1913. No capsules were given until Dec. 6. After that 1 gm. of the salt was given daily until Dec. 30. The dog became ill with an abscess below the right ear, from which he recovered, and fed well most of the time. The capsules did not seem to affect him. The animal died on Jan. 18, 1914, nearly 3 months after the beginning of the experiment. His weight varied according to his general condition, decreasing when the animal was ill, increasing so that at one time he gained 1 1/4 kilos, and at his death weighing about the same as when the experiment started. The total quantity of chondroitin sulphuric acid salt consumed was 47 gm. The cholesterin content of the blood appeared to be a little below the normal, being 0.001, while the normal for dogs is about 0.00137.[15] The autopsy showed a normal aorta and large vessels.

It was now proposed to feed dogs on bread and cottonseed oil, either without any, or with only a very small quantity of protein in the food. It was assumed at first that the animals could consume about 200 cubic centimeters of oil daily, but it was soon found that about 50 cubic centimeters was all that they could manage and keep in fair condition. The low protein diet was probably not necessary.

Dog 1.—Experiment commenced Nov. 8, 1913. The oil was rapidly increased to 200 c.c. per day. For the first few days the dog ate the food. After that he would eat only one third of his daily meal, and at times he would not touch the food at all. He was killed in a fight with another dog on Dec. 6, having lost about a kilo in weight.

The cholesterin content of the blood was not determined. Autopsy showed normal vessels.

[14] I am indebted to Dr. P. A. Levene of The Rockefeller Institute for Medical Research for placing a great quantity of the chemically pure soda salt of chondroitin sulphuric acid at my disposal.

[15] Dr. Edwin Henes, Jr. (Untersuchungen über den Cholesteringehalt des menschlichen Blutes bei inneren Erkrankungen, *Deutsch. Arch. f. klin. Med.*, 1913, cxi, 122) kindly made the cholesterin determinations for me.

Dog 2.—Experiment commenced Dec. 11, 1913. Bread, 2 oz. of meat, and 150 c.c. of cottonseed oil. The dog ate all the food for 3 days, after which he sometimes took one third, one half, or sometimes the whole. On Jan. 10, 1914, the oil was reduced to 50 c.c. From then on the dog ate all that was given him, till death on Jan. 21, 1914. His weight remained about the same, till the end, when he lost about 1 kilo.

The cholesterin content was 0.00225, almost double the normal. The autopsy showed pneumonia and a duodenal ulcer. There were a number of slight fatty streaks in the aorta.

Dog 3.—Experiment commenced on Nov. 29, 1913. Bread, 2 oz. of meat, 150 c.c. of oil, which were reduced on Jan. 6 to 50 c.c. From Dec. 23 to Jan. 28 meat was taken away altogether; the dog ate bread and oil only. It was attempted to make him run in the treadmill; for a number of days he could be made to work for an hour or even more. He developed an abscess on the jaw, so that he could run only at irregular intervals. Then convulsions set in, and the running had to be stopped altogether. He was killed on Jan. 31, the experiment lasting about 2 months. The weight increased slightly at first, then decreased somewhat, but at death was about the same as at the beginning, notwithstanding attacks of vomiting and diarrhea, besides convulsions.

The cholesterin content of the blood was 0.00228, also markedly above the normal. The anatomical diagnosis and autopsy showed adherent pericardium, dilatation of heart, fatty deposits in the liver and kidneys, pulmonary emphysema, intestinal parasites, and fine fatty striæ in the aorta.

URANIUM NITRATE AND OIL.

The oil feeding was then combined with uranium nitrate.

On Nov. 19, 1913, a dog was injected subcutaneously with 1 c.c. of a solution of uranium nitrate 0.5 to 100. The injection was repeated the next day. Typical symptoms of severe uranium nephritis followed. He was fed with oil soon reduced to 50 c.c., bread, and 2 oz. of meat. On Nov. 6 meat was taken away entirely, and the dog ate well most of the time. On Jan. 11, 1914, meat was given again. The animal died on Feb. 12, 1914, nearly 3 months after the beginning of the experiment. He received altogether 6 c.c. of uranium nitrate solution in five injections. The urine at death had a specific gravity of 1.018, albumin 0.3 per cent. (Esbach), some red cells, and numerous casts, mostly hyaline. He lost steadily in weight, altogether about 2,580 gm.

The cholesterin content of the blood was low, being only 0.00013. The autopsy showed typical uranium nephritis and infarction of lung. There were no changes in the aorta or large vessels.

A second uranium oil dog died eighteen days after the beginning of the experiment, without showing any special lesions besides nephritis.

CHOLESTERIN.

On January 27, 1914, an experiment was commenced with feeding cholesterin.

Dog 1.—The dog received bread, meat, 50 c.c. of cottonseed oil, and every other day a capsule of 0.5 gm. of pure cholesterin (Merck). It was not proposed to give the large doses of cholesterin which other investigators have given to rabbits and dogs, the idea being not to go beyond normal conditions more than was absolutely necessary. The animal took his food well until about the middle of Feb.; at that time the oil was stopped, and from Feb. 25 on he took 0.5 gm. of cholesterin every day with his bread and meat. Repeated attempts to make him run on the treadmill failed, on account of his feet becoming sore after running a day or two. After about 2½ months from the beginning of the experiment, the animal died on Apr. 8, 1914. The original weight of 6,700 gm. decreased steadily, so that at death he weighed only 2,400 gm. The autopsy showed pneumonia in the lungs; there were numerous small but quite distinct fatty patches and striæ in the aorta. The total quantity of cholesterin consumed was 25 gm. The cholesterin content of the blood serum was 0.001808.

Dog 2.—Feb. 5, 1914. Bread, 2 oz. of meat, 50 c.c. of oil, and 0.5 gm. of cholesterin were given every other day. Oil was stopped after 12 days. From Feb. 25 on 0.5 gm. of cholesterin was given daily. The dog ate well from the time the oil was stopped, and was found dead on Mar. 19, 1914. His weight had decreased about 900 gm. During the 1½ months he had consumed 16 gm. of cholesterin. The cholesterin content of the blood serum was 0.00196. The autopsy showed ascites, probably due to nephritis; there was a small, cartilagenous patch in the sinus above the posterior aortic cusp, surrounded by a yellowish area of hypertrophied intima; there were besides a number of hypertrophic spots in the thoracic aorta. The bile in the gall bladder contained large quantities of doubly refracting cholesterin crystals.

HYDROCHLORIC ACID.

A casual remark of Dr. P. A. Levene suggested the simple procedure of adding dilute hydrochloric acid to the dog's food and thus producing a chronic hyperacidity.

Dog 1.—Beginning Oct. 24, 1913, at first 20, after a few days 40, then 50, and finally 60 drops of dilute muriatic acid were added to the dog's ordinary food of bread and meat. The animal took the acid without any difficulty, and fed well till the last few days before death, which occurred on Dec. 13, 1913, about 2 months after the beginning of the experiment. Though the dog took all his food and seemed perfectly well, he nevertheless lost 3 kilos. The cholesterin content of the blood was not made in this case. The autopsy showed faint yellowish streaks just above the sinuses, behind the pulmonary cusps. The aortic cusps were normal. In the abdominal aorta, about 4.5 cm. above bifurcation, several raised patches of pinkish yellowish color were seen, somewhat irregular in shape, and with the surface wrinkled, the long axis parallel to the long axis of the

vessel; there were some patches also around the orifices of efferent vessels. Transverse ridges are marked in some of these patches, especially in several that are situated in the internal iliacs. Some similar patches are also recognizable in the carotids. There was considerable edema of the left lung, but no pneumonia. The cause of death is uncertain.

Dog 2.—Beginning Dec. 22, 1913, dilute muriatic acid was added to the food, so that after 21 days the daily quantity was 60 drops, which after that was not increased. The dog was killed on Mar. 25, 1914, 3 months after the beginning of the experiment. The animal was well during the entire time, and ate greedily. He was not full grown when the experiment was started, but grew steadily, and had gained three kilos when killed. The cholesterin content of the blood serum was 0.00159, just a trifle above the normal. At autopsy the dog was found to be well nourished, with good adipose. The heart contained numerous *Filaria mitis;* the endocardium of the left ventricle was somewhat milky. No valvular lesions were present. Immediately above the aortic cusps in the sinuses of Valsalva, also near the origin of the left carotid, and especially in the abdominal aorta, and there again most pronounced in its lower part, there were greyish yellow patches slightly raised above the surface of the intima. The largest patch was situated near the origin of the innominate artery, and around this the aorta is decidedly thin. The iliac arteries showed distinct transverse striation with yellowish grey and slightly raised mottling. There was nothing abnormal in the carotid and renal arteries. Otherwise there were no gross lesions.

DISCUSSION.

In reviewing briefly the outcome of all the experiments thus far reported, one fact stands out prominently; namely, that all the injection experiments failed absolutely to yield any positive result. Neither the poisoning with nicotin or lead, nor the infections with bacteria or bacterial products, appeared to afford the slightest evidence of atherosclerotic lesion in the aorta, or in any other vessel or organ of the dogs treated. If reasoning by analogy be permitted, it may be assumed that if a bacterial infection in man, which rarely lasts longer than a few weeks, is supposed to be an important etiological factor in subsequent atherosclerosis, then bacterial infections in dogs, prolonged for many months, and finally ending in death, might reasonably be expected to cause some atherosclerotic change.

In the case of the two dogs running in the treadmill, the objection might be raised that, though the strength of the animals was taxed to the utmost, the experiment did not last long enough to cause any damage. This objection, however, loses much of its weight when one finds from the last group of experiments that positive results can be brought about in time equally short or even shorter.

This last group of experiments, though still far from concluded, has already apparently furnished some points of interest. Chondroitin sulphuric acid does not seem to be effective, and does not require further discussion. All the other experiments, with the exception of two dogs to whom dilute hydrochloric acid was given, were undertaken for the purpose of studying the now generally conceded causal relation between cholesterin and the atherosclerotic process in man, as well as in the rabbit. Pure cholesterin (Merck) was given only in two cases. The other dogs took cottonseed oil, not only because this was less expensive than cholesterin, but also because it was thought probable that this oil contained sufficient lipoid material to answer every purpose. It was not proposed to administer the colossal quantities of cholesterin which some investigators had employed, and which exceeded by far all that either man or the rabbit would ever consume under natural conditions.

In all the oil and cholesterin dogs, with but one exception, atherosclerotic lesions were found. It is true, however, that only the very earliest stages appeared; *i. e.,* the fine yellowish striæ, and the fatty patches, in their gross appearance closely resembling the same structures so familiar in the human aorta.

The one exception was the dog with uranium nephritis. On general principles one would have expected that an oil-fed dog with chronic uranium nephritis, would be much more likely to develop atherosclerosis than a dog with sound kidneys. This seems not to have been the case. Neither in the aorta, the renal artery, nor in the kidneys, in fact in none of the vessels or organs of the nephritic dog, could any trace of atherosclerosis be found. The cholesterin content of the blood serum in this dog was much lower than in any other dog. It will be the subject for further study to arrive at an understanding regarding these phenomena.

In the aorta of the first acid-fed dog, as described above, there were found not only the fine, fatty striæ in the thoracic aorta, but quite large raised sclerotic patches in the lower portion of the abdominal aorta and in the external iliacs. These patches closely resembled those atherosclerotic areas in human beings that have not yet become ulcerated or calcified. This dog, however, was about four years old. The obvious objection was therefore close at hand

that the atherosclerosis in his case might have been spontaneous and entirely independent of the acid feeding. A young and growing dog was therefore subjected to the same treatment, and for a somewhat longer time, with the result that atherosclerotic changes similar to those in the first dog were now manifest over a considerably greater area of the aorta, both thoracic and abdominal, but more pronounced in the abdominal aorta. Though only two dogs have thus far been fed with hydrochloric acid, the possibility can not be denied, especially in view of the numerous negative results with other methods, that these positive results are not mere coincidences, but are probably due to the hydrochloric acid. Further investigations are necessary, and are under way.[16]

While writing this communication, the work of Oswald Loeb[17] has come to my notice. He describes results similar to mine in two dogs which he fed with large doses of lactic acid and kept on an exceedingly low protein diet. The detailed reports of his experiments, and especially the microscopic findings, have not yet been published. No diminished protein diet was given to our dogs. They fed well on their usual diet of bread and meat, thus showing that the sclerotic affection of the large vessels was in all likelihood due to the acid, and that it was probably immaterial whether the dogs ate meat or not.

The histological structure of this canine atherosclerosis has not yet been completely studied in all its details, but it has been ascertained that the lesions are primarily and principally localized in the intima. The intima of the dog's aorta, however, appears to differ somewhat in its structure from that as found in man and in the rabbit. It would appear that under normal conditions the elastica interna is covered by a single layer, possibly by several, fine layers of endothelial cells. Fibrous or muscular tissue is nowhere perceptible. The very earliest beginning of the sclerotic process is marked by a proliferation of rather large, flat cells, in all probability of en-

[16] Since writing the above, I have examined a third dog. The animal, scarcely one year old, died of pneumonia after having had acid feeding only for 3 weeks. He, too, showed a number of typical yellowish patches in the arch of the aorta.

[17] Loeb, O., Ueber experimentelle Arterienveränderungen mit besonderer Berücksichtigung der Wirkung der Milchsäure auf Grund eigener Versuche, *Deutsch. med. Wchnschr.*, 1913, xxxix, 1819.

dothelial origin. Notwithstanding protracted search, and aided by the best technical methods and all sorts of stains, nothing in any way resembling the macrophages or the large amebic leucocytes described by Saltykow could be found (figure 1). Almost simultaneously with the appearance of these large cells, elastic fibers begin to be split off from the elastica interna, and the whole process, as described by Jores, takes place (figure 2). A larger or smaller elevation or excrescence is thus formed, the base of which is still the elastica interna, and for some distance there can also be recognized one or two rows of those peculiar large endothelial-like cells, at the base of the elevation, and resting immediately upon the elastica. Between the newly split off elastic fibers which form, as it were, the skeleton of the little excrescence, there now develops a more or less swollen and succulent connective tissue, and possibly also some muscular fibers. In the meanwhile, and in that portion of the media underlying the newly formed elevated patch, the elastic fibers are beginning to give way, and the tissues there are also in a swollen and succulent state. No aggregation of leucocytes, or anything else pointing to an inflammatory process, could be made out. In some text-books it is stated that calcification has been found in the spontaneous atherosclerosis of dogs, and it is perhaps not improbable that calcification would soon have followed the developments just described, had the experiments lasted sufficiently long. Further investigation is required to clear up this point. It does not appear unreasonable to presume that the calcium metabolism in dogs, as well as in other omnivorous animals, differs from that of the rabbit, in which calcification, primarily in the media, occurs early and often spontaneously.

Simultaneously with the first appearance of the proliferating cells, and only among them and not in the normal intima, minute sudanophil droplets are seen contained in the upper portions of the proliferating cells, and also lying free above and between them. Very soon they are found in the media and down to the adventitia. As the process proceeds, the droplets become larger, sometimes run together, but never have we found them of the size and number as described in the rabbit and in man. They are most numerous in those fatty striæ and elevated fatty patches that we

found in the aortas of the oil-fed, and especially of the cholesterin-fed dogs (figure 3), but they also occur with great constancy, though much smaller in size, in the acid-fed dogs. They stain readily with Sudan III and Scharlach R; but they are optically inactive, and therefore are probably not cholesterin, but some other as yet undetermined fatty or lipoid substance. It is a striking fact that, while the bile of the cholesterin-fed dogs contained innumerable doubly refracting cholesterin crystals, not a single one could be found in the arteries, the liver, and the adrenals. Chalatow[18] has recently pointed out that the deposit of the doubly refracting so called fluid crystals, most abundant in the rabbit, does not take place in all animals. It would seem from the observations on our dogs, that, though the cholesterin content of the blood serum may be high, there is nevertheless no permanent deposit of cholesterin in the organs, and it is probable that there is a rapid elimination.

It is noteworthy, also, that the acid-fed dogs showed numerous sudanophil droplets in the early stages of the sclerotic hypertrophy; but that these sudanophil bodies had almost, if not entirely disappeared by the time the fully formed sclerotic area had developed.

The livers of all the dogs, as well as the adrenals, were crowded with sudanophil bodies. It could be shown that there was no fatty degeneration, but merely an overcrowding of the cells, and even to some extent of the intercellular tissue, with an infiltration of fatty material. Doubly refracting components could never be found. The livers of the acid-fed dogs, more than those of any of the other animals, were filled with this fatty substance, and it was especially the cylindrical epithelium of the bile ducts that was most conspicuously loaded with brilliant sudanophil matter (figure 4).

The work is being continued, and definite conclusions would at this stage be premature; but perhaps it may be permitted, even now, to venture the statement that in all probability the theory which bases atherosclerosis on a purely mechanical etiology will not prove tenable. Whether mechanical factors come into play at all, and if so, to what extent, remains to be seen.

It seems almost certain, at least in our present state of knowledge,

[18] Chalatow, S. S., Über flüssige Kristalle im tierischen Organismus, deren Entstehungsbedingungen und Eigenschaften, *Frankfurt. Ztschr. f. Path.*, 1913, xiii, 189.

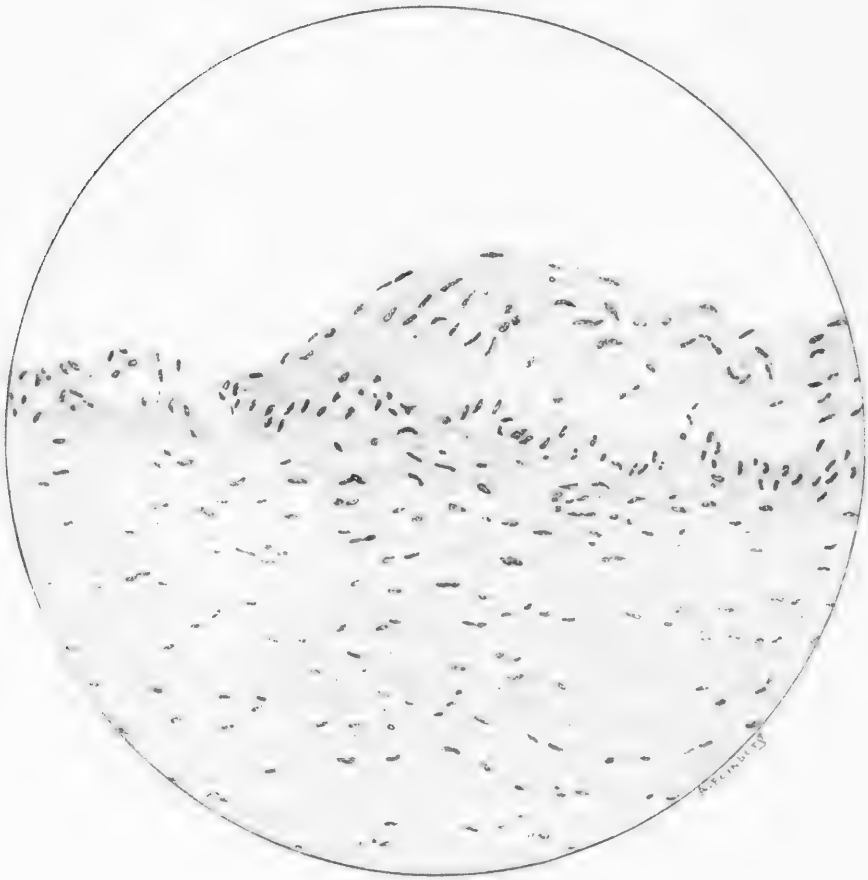

FIG. 1.

(Adler: Studies in Experimental Atherosclerosis.)

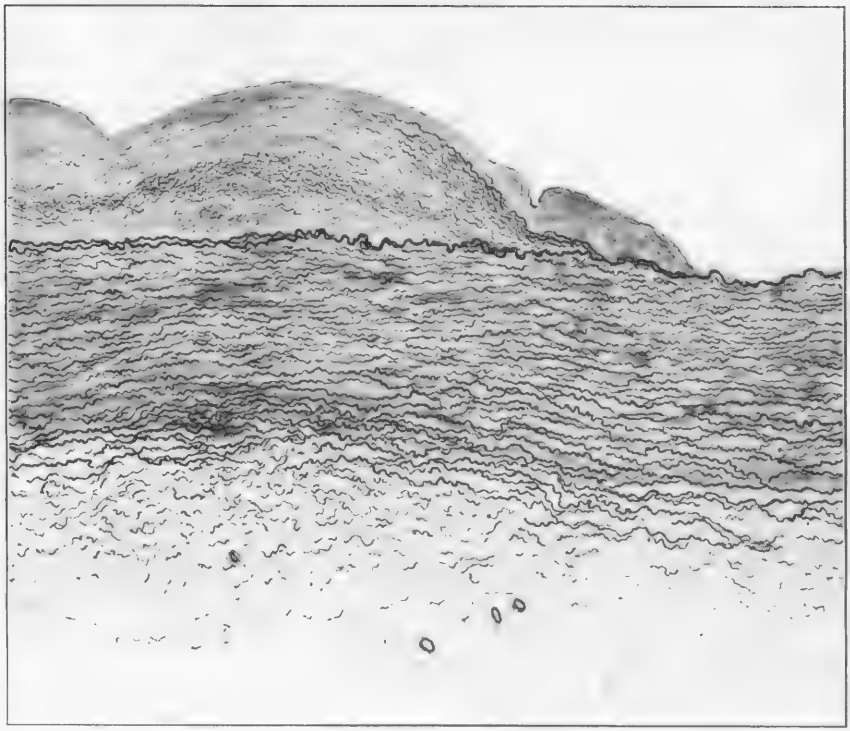

FIG. 2.

(Adler: Studies in Experimental Atherosclerosis.)

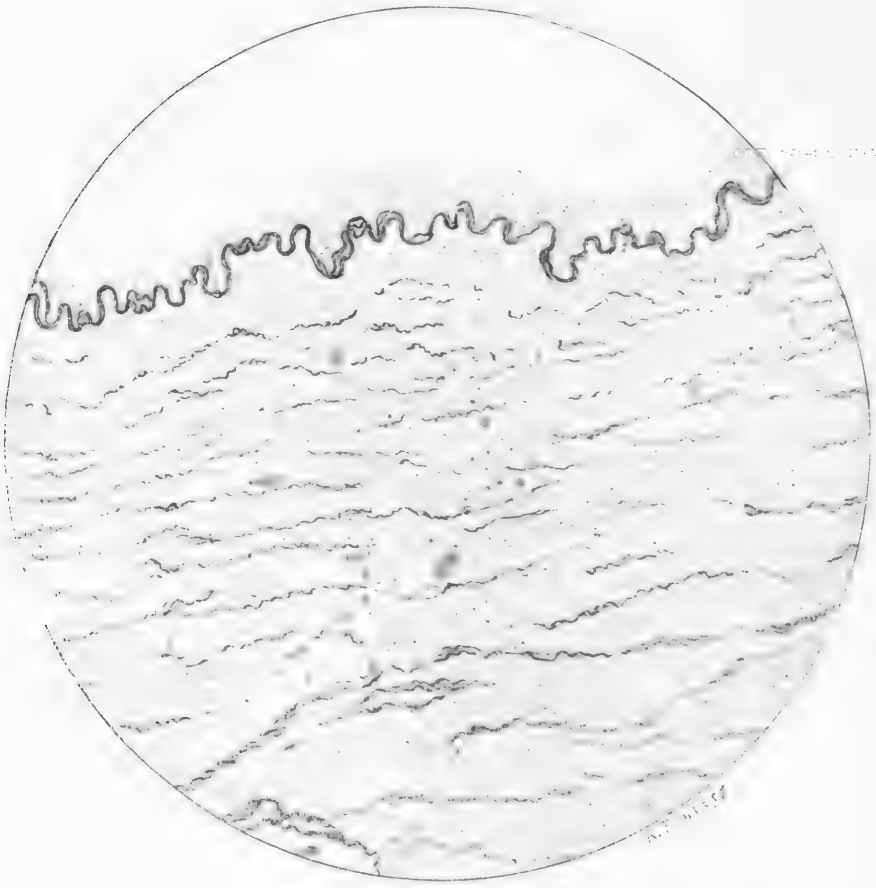

FIG. 3.

(Adler: Studies in Experimental Atherosclerosis.)

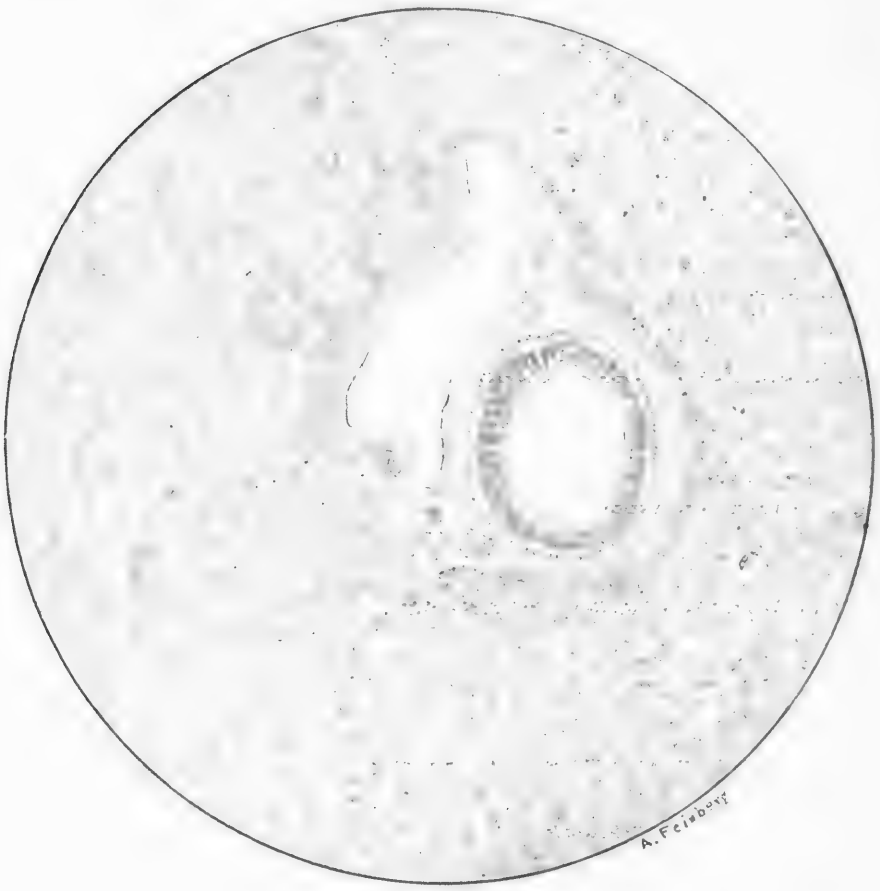

FIG. 4.

(Adler: Studies in Experimental Atherosclerosis.)

that chemical influences, subject possibly to more or less nerve control, are dominant factors in the etiology of atherosclerosis. Perhaps it may be discovered also that cholesterin and its various modifications and combinations, while undoubtedly an element of importance in atherosclerosis of the rabbit and human beings, may not be the sole predominant etiological factor. If it should turn out that so simple a procedure as adding a certain proportion of hydrochloric acid to the food of dogs is sufficient to produce lesions of the blood vessels closely analogous, if not wholly identical with human atherosclerosis, a revision of our present theories will become necessary.

EXPLANATION OF PLATES.

Plate 7.

Fig. 1. Section through a sclerotic patch from the aorta of an acid-fed dog. Weigert-Van Gieson stain shows the proliferation of endothelial cells and the typical hypertrophy of the intima.

Plate 8.

Fig. 2. Section through the same aorta. Weigert elastic stain shows splitting off of elastic fibers from the elastica interna.

Plate 9.

Fig. 3. Frozen section through a yellow patch of the aorta of an oil-fed dog. Sudan III shows incipient proliferation of the endothelium and distribution of sudanophil bodies.

Plate 10.

Fig. 4. Frozen section through the liver of an acid-fed dog. Stained with Sudan III. The liver cells and especially the cells of the bile ducts are crowded with sudanophil bodies.

THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

X. Concerning the Supposed Regulatory Influence of the Spleen in the Formation and Destruction of Erythrocytes.

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In the older literature of the physiology of the spleen appear numerous references to differences in the composition of the blood of the splenic artery as contrasted with that of the splenic vein, and in the recent literature observations are made upon the occurrence of a specific hemolysin in extracts of splenic pulp. The possible bearing of these observations upon the various studies[1] of the spleen reported from this laboratory during the past two years led to a critical examination of the general literature and to certain experiments already described, in the hope that light might be thrown upon some of the difficult aspects of the general problem. A careful study showed that the observations in question are subject to a large experimental error which renders the finding of slight differences in the composition of arterial and venous blood of doubtful value,—a reason why the work has not heretofore been utilized in our reports. Indeed the work probably would have been entirely disregarded if Banti had not recently claimed, in support of his theory of the splenic origin of icterus, that the blood of the splenic vein normally contains more free hemoglobin than the blood of the general circulation. In order to test this and other points we have repeated experiments upon which the assumption that the spleen has a direct or indirect influence upon the red cells is based.

* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, May 12, 1914.

[1] *Jour. Exper. Med.*, 1912, xvi, 363, 375, 758, 769, 780; 1913, xviii, 487, 494, 665; 1914, xx, 19.

Much of the early work on this subject is not only contradictory but was done before the development of the present exact methods of blood examination. Thus Virchow (1) found fewer red cells in the blood of the splenic vein than in that of the artery; while Malassez and Picard (2) and Emelianow (3) report the opposite. On the other hand, later investigators, Vulpius (4) and Paton, Gulland, and Fowler (5), have found no constant or noteworthy differences.

Considering the spleen as a possible leucoblastic organ, numerous early observers (6) found relatively more leucocytes, especially so called young forms, in the blood emerging from the spleen, than in that entering it. Tarchanoff and Swaen (7) and also Virchow (1) could not find any noteworthy difference, whereas Paton, Gulland, and Fowler (5) noted a constant diminution in the number of leucocytes in the splenic vein as compared with the general circulation. In this connection Bulgak (8), who describes an increase in leucocytes in the splenic vein, states that this is true of the venous blood of all parenchymatous organs. Freyer (9) concludes from his comparative counts that the spleen has nothing to do with blood formation.

The preceding studies refer of course to mature animals. It is accepted that in fetal life the spleen has the power of extensive blood formation, and several reports are at hand to show that the adult spleen may undergo, in the presence of injury to the bone marrow, a myeloid metaplasia (10); that is, that it can regain under pathological conditions its fetal function. Whether or not the spleen may exert this power of blood formation in the adult under normal conditions is doubtful, though still an open question.

Although the spleen certainly destroys red blood cells, as is evident from the presence in it of large cells, phagocytic for erythrocytes, which are increased under certain pathological circumstances, there still exists doubt whether the destruction by phagocytosis is the only method of red cell disintegration. It is stated also that the erythrocytes, in their passage through the spleen, are so acted upon by some unknown substance as to become more susceptible to hemolysis. This is the basis of Bottazzi's (11) hemocatatonistic theory, which has recently received support from Banti (12) and his colleague Furno (13). In the course of an investigation of hemolytic splenomegaly, they studied normal animals and those receiving hemolytic serum and came to the conclusion that free hemoglobin can be demonstrated in the blood of the splenic vein in normal animals as well as in animals receiving hemolytic serum. It was found at times in the blood of other vessels but in less amounts than in the splenic vein. They consider the findings as evidence of hemolysis in the spleen. The red blood cells of the splenic vein were found also to be less resistant to hypotonic salt solution than were those of the general circulation. Observations by Chalier and Charlet (14) on the resistance of red cells in the splenic artery and vein gave different results. Although the venous blood in general was slightly less resistant than arterial blood, this was reversed in the splenic system, so that the blood of the splenic vein was more resistant than that of the splenic artery and much more than the blood of other veins. Hammarsten is also said by Gabbi (15) to have found that the splenic vein blood was more resistant than the arterial. In the observations of Banti and Furno, the reference is to free hemoglobin in the serum and not to the increased hemoglobin content of venous or splenic blood described by several investigators (16).

The claim of Banti and Furno is surprising in that they state that the dissociated hemoglobin of the serum (*"emoglobin disciolta dal siero"*) is not only always present in the splenic vein of normal animals, but in sufficient quantities sometimes to be measured by a Sahli hemoglobinometer. It is to these observations that we have given especial attention in our work.

COMPARISON OF THE ARTERIAL AND VENOUS BLOOD.

Method.—From dogs under ether anesthesia, blood was obtained directly from the splenic artery and the splenic vein. Great care was exercised to disturb the vessels and the organ as little as possible, as it has been shown by Grigorescu (6) and Pribram (17) that the cell content of the blood may be greatly increased by congestion of the spleen. From a nick in the vessel wall of one of the branches of the artery or vein, fresh blood was drawn directly into Thoma blood-counting pipettes and the capillary tube of a von Fleischl hemoglobinometer. From another branch, blood was withdrawn by a syringe and immediately distributed to tubes containing different strengths of hypotonic salt solution designed to test the resistance of the red cells. Some of the blood was also set aside for similar tests with washed cells. For the determination of the presence of free hemoglobin in the serum, blood was collected in three ways: (1) in a paraffined centrifuge tube, (2) in a tube containing potassium oxalate, and (3) by drawing it directly into tubes through capillary points which were then sealed. All three samples were then centrifuged and the serum was examined for hemoglobin by visual inspection and the spectroscope. Smears for differential counts were made at times from the blood flowing directly from the vessel and at times from a drop from the syringe. Finally, tests for reticulated or skeined (young) red blood cells were made. This was done by letting a few drops of blood fall into a solution of brilliant cresyl blue, and, after standing fifteen or twenty minutes, the skeined forms in proportion to the unskeined or mature forms were counted in fresh smears. For the purpose of controls, blood from the femoral vein, and from the capillary circulation by puncture of the skin was occasionally collected.

Results.—The figures for the red and white cells, differential counts, and total hemoglobin in a series of five dogs show that as far as these estimations are concerned the blood of the splenic vein does

not differ greatly from that of the artery. The variations are not uniformly on one side and are all within the limit of error inherent in the methods of blood examination.

It is of interest that in these and other dogs, the red cells of the vein, in six of eight animals, showed more or less marked anisocytosis and inequality of staining, which were not seen to the same degree in the blood of the artery. Polychromatophilia was about equal in artery and vein. In two of the eight animals a few normoblasts were found in the splenic vein blood only. Control smears from the femoral vein of four dogs showed changes in the red cells about equal to that of the splenic vein, indicating that these changes are characteristic of venous blood in general rather than a specific change caused by passage through the spleen.

In regard to the presence of free hemoglobin in the serum, if we had depended on one tube only, we should have occasionally found apparent hemoglobinemia, both of the general circulation and of the splenic vein; but as in every set of three tubes, in a series of seven dogs, at least one was free of hemoglobin, we cannot support the view that free hemoglobin in demonstrable amounts is present normally either in the splenic vein or in the general circulation of the dog. Our experience forces us to the conclusion that the findings of other investigators are due to hemolysis after collection or are dependent upon the method of separating the serum.

As regards the resistance of the red cells, of which comparative tests were made on eight dogs, in five no difference was found between artery and vein; in the other three, the venous corpuscles were slightly less resistant. Two control tests with cells from the femoral vein showed these to have the same resistance as those of the splenic vein.

In seven comparative tests for skeined or reticulated red corpuscles, these were found five times to be more abundant in the splenic vein and twice more numerous in the artery; the differences were never very striking. Five controls from the femoral vein corresponded more closely to the splenic artery counts than those of the splenic vein.

Conclusions.—As a result of the various observations we conclude that the slight differences between the arterial and venous

blood of the spleen are within the limits of error inherent in the methods of blood examination and are not to be explained by a peculiar action of the spleen. In some instances peculiarities shown by the splenic venous blood are common to the venous blood of the general circulation. Banti and Furno's observation concerning the presence of free hemoglobin in the blood of the splenic vein is not confirmed.

THE HEMOLYTIC POWER OF SPLENIC EXTRACTS.

The histological evidence of the destruction of erythrocytes by phagocytic cells of the spleen has naturally suggested the possibility of the liberation by these cells of a ferment capable of acting extracellularly. If such a free hemolysin is present in the spleen it should be demonstrable in extracts of the spleen, and during the past few years several investigators have therefore tested the influence of such extracts upon red cells. The methods employed, based on the technique of Korschun and Morgenroth (18), are similar, but the results obtained have been contradictory.

Korschun and Morgenroth found in several organs a hemolytic substance of unknown origin, coctostabile and soluble in alcohol, which did not arise from constituents of the blood serum and was in no way peculiar to the spleen. Nolf (19), on the other hand, found that the hemolytic power of splenic extract was distinctly greater than that of the liver, mesenteric lymph nodes, or kidneys, but only slightly more than that of the lung. This hemolytic substance was specific for the species and was destroyed at 100° C. Achard, Foix, and Salin (20), repeating these experiments, showed that the final solution was strongly acid, presumably as the result of bacterial action, and that control tests made with precaution as to asepsis were uniformly negative. Widal, Abrami, and Brulé (21) in similar experiments could get no hemolysis with fresh extracts used on the day they were prepared; sometimes, also, extracts twenty-four to forty-eight hours old were without effect. From these results they conclude that the hemolytic substance is not a true hemolysin, but the product of cell autolysis. Iscovesco and Zacchiri (22) had previously shown that after placing the mixture of pulp and saline solution in the thermostat for fifteen to twenty hours, the filtered extract, on the addition of red cells and after standing two and one half hours in the thermostat, showed 2.5 to 8 per cent. hemolysis, as determined by the Dubosc colorimeter, and they conclude that the hemolytic power of splenic extracts is unimportant. Weill (23) found a weakly hemolytic substance in extract of spleen that was inactivated at 56° C. and reactivated with guinea pig serum. This was more powerful than a lymph node extract prepared in the same way, but much less powerful than the extract obtained from the spleen by long maceration. The latter was not destroyed below 80° C., and its action was hindered by adding

fresh serum. Extracts from lymph nodes prepared in the same way showed only slight hemolytic action and those from other organs were negative. Banti (12) and Furno (13) state that fresh extracts of the normal spleen sometimes have no hemolytic action, and sometimes a weak action which is increased on standing twenty-four to forty-eight hours on ice and is not destroyed by heating to 60° or even to 100° C. They consider it a cytohemolysin, normally present in the spleen in small amounts and much increased after the administration of hemolytic agents. Thus we find that Nolf, Weill, Banti, and Furno find splenic extracts to have a hemolytic action greater than that of other organs. Achard, Foix, and Salin, and Widal, Abrami, and Brulé, on the other hand, fail to find any hemolytic action of the fresh extract, and think it is found only after autolysis or bacterial decomposition of the spleen.

Our experiments were made with extracts from the spleens of three dogs. The technique described by Nolf was followed in the main with several additions in the way of control experiments. On washing through the aorta, it was found that the technique which will give a blood-free kidney or liver will not render the spleen bloodless. Various expedients were tried, therefore, to secure a hemoglobin-free extract. It was found that if the spleen, after washing through the aorta, was cut in small pieces and pounded with a pestle against a wire-meshed sieve placed in a mortar, with the aid of frequent washings with salt solution, a blood-free white mass was obtained consisting partly of reticulum and partly of adherent splenic pulp (extract A, table I). As it was possible that the hemolytic substance might not be retained, or in only small amounts, in this fraction, extracts were also made from that part of the spleen that was mashed through the sieve. This residue was of course distinctly blood-tinged, so that it was necessary, in order to remove the blood, to mix it with distilled water, centrifuge, discard the supernatant fluid, and repeat the process until colorless tissues were obtained (extract B, table I). In each case the material thus obtained was mixed with double the amount of salt solution and placed in the refrigerator. Tests were always made with extracts one or two hours old, a small portion being filtered off for this purpose, and in two instances also after eighteen and twenty-four hours. Control tests were made in one experiment with extracts of liver and mesenteric lymph nodes. As it was possible to wash the latter free of blood before removal from the body, an extract was easily obtained by grinding the tissue in sand with mortar

TABLE I.

Character of extract.	Amount of splenic extract in c.c.								Salt solution control.	Distilled water control.
	1.95	1.5	1.0	0.5	0.3	0.2	0.1	0.05		
1. Dog 1. Fresh spleen extract A.	—	V.S.	0	0	0	0	0	0	0	C.
2. Dog 2. Fresh spleen extract A.	—	0	0	0	0	?	0	0	0	C.
3. Same. Extract B.	—	0	0	0	0	0	0	0	0	C.
4. Same. After extraction in ice chest for 24 hrs.	—	V.S.	V.S.	0	0	0	0	0	0	C.
5. Dog 3. Fresh spleen extract A.	V.S.	0	0	0	—	0	0	0	0	C.
6. Spleen extract A after extraction in ice chest for 24 hrs.	—	0	0	0	0	0	0	0	0	C.
7. Fresh spleen extract (boiled).	0	0	0	0	—	0	0	0	0	C.
8. Fresh liver extract.	0	0	0	V.S.	—	0	0	0	0	C.
9. Fresh mesenteric lymph node extract.	0	0	0	0	—	V.S.	0	0	0	C.
10. Mesenteric lymph node extract after extraction in ice chest for 24 hrs.	—	0	0	?	V.S.	M.	V.S.	0	0	C.

0 = no hemolysis; ? = doubtful hemolysis; V.S. = very slight hemolysis; M. = marked hemolysis; C. = complete hemolysis; — = no test.

and pestle and placing it as before in the ice chest with double the amount of salt solution. In two experiments the tests were made on the corpuscles of the animal furnishing the spleen; in one the corpuscles of another dog were used without a difference in result. The preparation of the washed red blood corpuscles, the dilutions, incubation, and so forth, were made according to Nolf's technique. Each tube contained 0.1 of a cubic centimeter of washed dog's corpuscles with varying amounts of splenic extract made up to two cubic centimeters with normal salt solution. Controls were made with normal salt solution and distilled water. The results are presented in table I.

Conclusions.—Fresh extracts of spleen are devoid of definite hemolytic action. Occasional trivial and irregular results, not to be explained, are found, but these occur likewise in the control extracts of liver and mesenteric lymph nodes. Extracts twenty-four hours old, prepared at low temperature, show little or no increase in hemolytic activity. Boiled splenic tissue, extracted in the cold for twenty-four hours, is inert.

THE INFLUENCE OF THE INTRAPERITONEAL INJECTION OF SPLENIC EXTRACT.

The changes in the blood picture following experimental removal of the normal spleen suggest that changes of interest might be produced by a converse procedure; namely, introduction into the body of the products of splenic activity in the form of splenic extract. If temporary anemia follows removal of the spleen, one might expect that some temporary rise in the red cell count might follow the injection of splenic extract.

The literature concerning the spleen contains very few reports on this subject. Danilewsky (24) found a surprising increase in hemoglobin and red blood cells after a single subcutaneous or intraperitoneal injection of extract of spleen. This increase reached its height in from three to seven days and continued as long as the experiment lasted, usually eight days. In dogs with a dietary anemia, splenic extract caused an even greater rise; for example, of 40 per cent. hemoglobin and almost 2,000,000 red cells. Danilewsky assumed that his results were due to a stimulation of the bone marrow. This influence of the splenic extract was not destroyed by heating.

Danilewsky's work, however, is uncontrolled by injection of other organ extracts, and the rise noted extended over a surprisingly long period of time. Silvestri (25) records a single observation in which a dog, presumably dying from anemia, was apparently saved by the injection of splenic extract. In this connection it must also be noted that the clinical literature of this subject contains several reports (26) of the use, with good results, of extracts of spleen and bone marrow in the treatment of anemia.

Method.—We have tested the effect of splenic extract on four dogs, using as controls extracts of other organs similarly prepared and extracts of erythrocytes.

The usual examinations of the blood were made, and also determinations of the resistance of the erythrocytes to hypotonic salt solution and the percentage of skeined cells. As a rule two counts were made before injection, and daily counts after the injection until the blood picture had returned to normal, usually a period of from three to four days. Extracts were prepared from organs removed aseptically from dogs bled to death under ether anesthesia. The finely chopped organ was ground in a sterile mortar to a homogeneous pulp and extracted with double the volume of salt solution for two hours in the ice chest. Ten cubic centimeters of the filtered extract were injected intraperitoneally into dogs of about the same

weight. Defibrinated blood diluted 1 to 20 with normal salt solution was used in ten cubic centimeter amounts to control the possibility of the rise in red cell count being due to the influence of some constituent of the red cells. In no case did peritonitis or other infection result. The result of one of these experiments is shown in table II.

TABLE II.

Date (1914).	Hemoglobin.	Red blood cells.
Feb. 6	102	5,250,000
Feb. 7	101	5,650,000
	(10 c.c. splenic extract No. 16 injected.)	
Feb. 8	110	6,500,000
	(15 c.c. of same extract injected.)	
Feb. 9	110	7,040,000
Feb. 10	105	6,800,000
Feb. 11	96	5,330,000
Feb. 12	95	5,290,000
	(15 c.c. splenic extract No. 88 injected.)	
Feb. 13	101	5,700,000
	(10 c.c. of same extract injected.)	
Feb. 14	104	6,880,000
Feb. 15	98	5,860,000
Feb. 16	96	5,120,000
Feb. 19	106	5,540,000

This experiment shows that intraperitoneal injection of splenic extract causes a sharp rise in hemoglobin and red cell count, lasting only one or two days. This rise is repeated on reinjection of either the same or another splenic extract.

In each of three other experiments with splenic extract an increase in the number of red cells was obtained, but this increase was not always as marked as in the experiment presented; it was nevertheless always greater than that caused by the use of control extracts of liver, kidneys, or blood.

The study of the resistance of the red cells may be dismissed with the statement that no noteworthy differences were found after injection of any extract. The skeined cells also showed no constant change. We had hoped that as the latter are supposed to be young forms of erythrocytes, they would be found to be increased after the injection of splenic extract had caused a rise in the red cell

count. Only once, however, when the percentage rose from 0.5 to 2 per cent., was this noticed. On the other hand, in two experiments they were not found at all in the blood after injection.

Intraperitoneal injection of splenic extract is usually followed by an increase in the total number of leucocytes, consisting chiefly of the polymorphonuclear forms. A similar rise occurred in one of three injections of liver and kidney, and in one of two of defibrinated blood. Several grades of transitional cells appeared in increased numbers. Eosinophils were present in increased numbers in two of the four dogs receiving splenic extracts, but were also definitely increased in two of the five controls.

Conclusions.—Intraperitoneal injection of saline extracts of fresh spleen constantly causes a sharp increase in red cell count and hemoglobin content. The rise is evanescent, lasting but one or two days, and may be followed by an equally evanescent drop below normal. Similarly prepared extracts from other organs fail to give this rise. No noteworthy change is found in the resistance of the red blood cells to hypotonic salt solutions, or in the number of skeined or reticulated erythrocytes, after the injections of the various organ extracts.

A temporary increase of polymorphonuclear and transitional leucocytes usually follows the use of spleen extract, but may occur also, though less frequently, after the injection of liver and kidney.

The constant increase of red cells in the peripheral circulation after injection of spleen, in view of the tendency to anemia following splenectomy, suggests that the spleen normally may exert a stimulating effect upon the formation of red cells in the bone marrow.

THE INFLUENCE OF FEEDING SPLEEN TO SPLENECTOMIZED DOGS.

This study complements that just described in that spleen in large amounts was fed to splenectomized animals. The object was to determine whether through the influence of a possible internal secretion of the spleen the anemia following splenectomy might be prevented. The procedure is of course analogous to thyroid feeding in insufficiency of the thyroid gland, and has an advantage over the injection of extracts in that it may be continued over long periods of

time without the possibility of the complications occasionally occurring after injection. These experiments, it was hoped, would show whether or not the spleen exerts some effect upon the hemopoietic system through peculiar bodies concerned perhaps in an internal secretion. Thus, if the anemia following splenectomy depends upon the absence of a normal stimulus to the hemopoietic system in general, or to some part of it, as the bone marrow, furnished normally by the spleen, the feeding of normal fresh spleen unmodified by heat or chemicals would supply this secretion and there would be no anemia after the removal of the spleen.

Method.—Five dogs were used. Four of these were given a diet consisting of raw hashed beef spleen, lard, and cracker meal in amounts estimated, according to the weight of each animal, to suit its caloric needs. Of these, three were splenectomized and one served as a control. As an added control, a splenectomized dog received a diet in which casein was substituted for beef spleen. The red cells and the hemoglobin were estimated several times before splenectomy and afterwards counted twice a week for three weeks. After this they were counted every week for approximately five weeks. No preliminary counts were made until a dog had been on the special diet for at least a week, and splenectomy was not performed until two weeks later.

Results.—Of the three splenectomized dogs receiving spleen in the diet, one showed a very slight decrease in red cells and hemoglobin, but the other two developed the usual anemia of splenectomy. Thus one with an initial red cell count of 6,200,000 showed on the 12th day 4,710,000 red cells with return on the 54th day to 6,040,000. This animal received daily 150 grams of beef spleen. The other dog receiving daily 275 grams of spleen showed a change in red cell content of about the same degree. In the splenectomized dog not fed spleen, the red cells fell from 5,550,000 to 4,210,000 on the 19th day with return to 5,060,000 on the 54th day. In this dog the hemoglobinemia reached its lowest level (65 per cent.) on the 12th day, and remained at about that point until the 26th day. In neither of the other splenectomized animals receiving spleen did the hemoglobin fall below 75 per cent. The normal dog, receiving 150 grams of spleen daily, showed no change in the blood.

It is evident that in two dogs, despite the feeding of spleen, an anemia was produced that ran a course similar to that which we have previously shown to be the rule in splenectomized dogs (27). In view of these definite results, the absence of marked anemia in the third splenectomized dog must be considered as the result of factors other than the feeding of spleen.

Incidentally it was found that the resistance of the red cells to hypotonic salt solution was increased in all splenectomized dogs, thus confirming the work of Karsner and Pearce (28).

Conclusion.—The feeding of fresh raw spleen to splenectomized dogs has no clearly defined influence in preventing the anemia which usually occurs after splenectomy.

GENERAL SUMMARY.

1. The blood of the splenic artery and vein shows either no differences, or only such slight irregular variations as may be due to the errors inherent in hematologic methods, or are common to arterial and venous blood of the general circulation.

2. The observation of Banti and Furno that free hemoglobin occurs in the blood of the splenic vein is not confirmed.

3. Extracts of the spleen have no definite hemolytic action *in vitro*.

4. Intraperitoneal injection of fresh saline extracts of the spleen causes in the dog a sharp increase in the number of red cells and the hemoglobin content which lasts for one or two days and may recur on a second injection. Extracts of liver, kidney, and erythrocytes similarly prepared do not give this effect. This observation supports Danilewsky's theory that the spleen may exert a stimulating effect upon the formation of red cells in the bone marrow.

5. On the other hand, the feeding of raw beef spleen to splenectomized dogs over long periods of time has no clearly defined influence in preventing the anemia which usually follows splenectomy.

BIBLIOGRAPHY.

1. Virchow, R., Zur pathologischen Physiologie des Bluts. Die Bedeutung der Milz- und Lymphdrüsen-Krankheiten für die Blutmischung, *Virchows Arch. f. path. Anat.*, 1853, v, 43.

2. Malassez and Picard, Recherches sur les fonctions de la rate, *Compt. rend. Acad. d. sc.,* 1875, ii, 984; La splénotomie et l'énervement de la rate, *Gaz. méd. de Paris,* 1878, vii, 185.
3. Emelianow, P., Sur le rôle de la rate au point de vue de la composition morphologique du sang, *Arch. d. sc. biol.,* 1893, ii, 135.
4. Vulpius, O., Beitrag zur Chirurgie und Physiologie der Milz, *Beitr. z. klin. Chir.,* 1894, xi, 633.
5. Paton, D. N., Gulland, G. L., and Fowler, J. S., Relationship of the Spleen to the Formation of the Blood Corpuscles, *Jour. Physiol.,* 1902, xxviii, 83.
6. Funke and Hirt, cited by Tarchanoff, J., and Swaen, A., Des globules blancs dans le sang des vaisseaux de la rate, *Arch. de physiol. norm. et path.,* 1875, ii, 324. Gibson, L., Blood-Forming Organs and Blood Formation; an Experimental Research, *Jour. Anat. and Physiol.,* 1885, xx, 100. Grigorescu, Modifications du sang par le séjour prolongé expérimentalement dans la rate, *Compt. rend. Soc. de biol.,* 1887, xxxix, 548; Sur le rôle hémopoiétique de la rate, *Arch. de physiol. norm. et path.,* 1891, iii, 561. Emelianow, P., *loc. cit.*
7. Tarchanoff, J., and Swaen, A., *loc. cit.*
8. Bulgak, J., Ueber die Contractionen und die Innervation der Milz, *Virchows Arch. f. path. Anat.,* 1877, lxix, 181.
9. Ouskow, N., and Sélinow, A., De la rate suivant les globules blancs du sang et le nombre de ces derniers, *Arch. d. sc. biol.,* 1897, v, 1.
10. Donhauser, J. L., The Human Spleen as an Haematoplastic Organ, as Exemplified in a Case of Splenomegaly with Sclerosis of the Bone Marrow, *Jour. Exper. Med.,* 1908, x, 559.
11. Bottazzi, F., La milza come organo emocatatonistico, *Sperimentale,* 1894, xlviii, 433.
12. Banti, G., La splénomegalie hémolytique, *Semaine méd.,* 1912, xxxii, 265; La splenomegalia emolitica anemopoietica. (Anemia emolitica splenomegalica anemopoietica) ufficio della milza nell' emolisi, *Sperimentale,* 1913, lxvii, 323.
13. Furno, A., Ricerche sperimentali, ematologiche ed anatomopatologiche intorno all' emolisi da siero negli animali normali e splenectomizzati, *Sperimentale,* 1913, lxvii, 639.
14. Chalier, J., and Charlet, L., État de la résistance globulaire chez l'animal normal et splénectomisé, *Jour. de physiol. et de path. gén.,* 1911, xiii, 728.
15. Hammarsten, cited by Gabbi, U., Die Blutveränderungen nach Extirpation der Milz, in Beziehung zur hämolytischen Function der Milz, *Beitr. z. path. Anat. u. z. allg. Path.,* 1896, xix, 647.
16. von Middendorf, M., Inaugural Dissertation, Dorpat, 1888; Glass, V., Inaugural Dissertation, Dorpat, 1889; Darjewitsch, C., Inaugural Dissertation, Dorpat, 1889; cited by Meyer, A., Beitrag zur Kenntnis der Milzfunktion, *Centralbl. f. d. Grenzgeb. d. Med. u. Chir.,* 1914, xviii, 41.
17. Pribram, B. O., Hypersplenische Hämophthisen und Stauungsmilz, *Wien. klin. Wchnschr.,* 1913, xxvi, 1607.
18. Korschun, S., and Morgenroth, J., Über die hämolytischen Eigenschaften von Organ-Extracten, *Berl. klin. Wchnschr.,* 1902, xxxix, 870.

19. Nolf, P., Le pouvoir autohémolytique du suc de rate, *Compt. rend. Soc. de biol.*, 1912, lxxii, 121.
20. Achard, C., Foix, C., and Salin, H., Sur le pouvoir hémolytique de l'extrait de rate, *Compt. rend. Soc. de biol.*, 1912, lxxii, 394.
21. Widal, F., Abrami, P., and Brulé, M., A propos du rôle hémolytique de la rate normale, *Compt. rend. Soc. de biol.*, 1912, lxxii, 694, 732.
22. Iscovesco, H., and Zacchiri, E., Sur le pouvoir autohémolytique de la rate, *Compt. rend. Soc. de biol.*, 1911, lxxi, 702.
23. Weill, O., Hémolyse locale et hémolyse splénique, *Trav. du lab. de l'Inst. Solvay*, 1913, xii, 180.
24. Danilewsky, B., Über die blutbildende Eigenschaft der Milz und des Knochenmarks, *Arch. f. d. ges. Physiol.*, 1895, lxi, 264.
25. Silvestri, T., Milza e eritropoiese, *Pathologica*, 1913, v, 145.
26. Pitts and Ballance, Splenectomy for Rupture, *Lancet*, 1896, i, 484.
27. Musser, J. H., Jr., and Krumbhaar, E. B., The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. VI. The Blood Picture at Various Periods after Splenectomy, *Jour. Exper. Med.*, 1913, xviii, 487.
28. Karsner, H. T., and Pearce, R. M., The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. IV. A Study, by the Methods of Immunology, of the Increased Resistance of the Red Blood Corpuscles after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, 769.

THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

XI. The Influence of the Spleen on Iron Metabolism.*

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This investigation was undertaken to determine whether the tendency to anemia in splenectomized dogs and the delayed regeneration of the blood after the administration of hemolytic agents to such dogs[1] might be due in part to some influence of the spleen upon the iron metabolism, as has been claimed by Asher (1).

Our present knowledge concerning iron metabolism may be summarized as follows: Iron is absorbed only to a very limited extent from the gastro-intestinal tract, so that when abundant in the food it passes for the most part unchanged from the intestine in the feces. As much as is absorbed is taken up chiefly from the small intestine and carried by the lymph to be deposited in the liver and to a lesser extent in the spleen, bone marrow, and perhaps elsewhere, and this occurs whether the iron be in intimate organic combination, the so called food iron, incapable of giving the characteristic microchemical reaction, or whether it be in the form of an organic or inorganic salt of iron. Moreover, from the work of Häusermann (2) and of Abderhalden (3), it appears that though iron salts are absorbed, the body is unable, or but very poorly able, to utilize them for the building of hemoglobin, being dependent for this constructive work upon the intimately combined food iron. On the other hand, iron salts are effective stimulants to the blood-

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[1] *Jour. Exper. Med.*, 1912, xvi, 363, 375, 758, 769, 780; 1913, xviii, 487, 494, 665; 1914, xx, 19, 108.

forming organs and conspicuously increase the utilization by them of the food iron.

The elimination of iron occurs almost wholly through the intestines, especially the colon, the quantity passing out in the urine constituting less than 1 per cent. of the total excretion in man and the dog. In the fasting dog the output found by von Voit (4) was 0.60 of a milligram per kilo of body-weight per day, and on an adequate, but iron-poor diet, Gottlieb's (5) dog excreted 0.34 of a milligram. For man the figures are lower. Cetti and Breithaupt (6), while fasting, eliminated about 0.10 to 0.13 of a milligram per kilo per day; and in various studies on man 0.10 to 0.25 of a milligram per kilo per day have been found to be the intake required to maintain iron equilibrium. However, there is every reason to believe, as is suggested by the work of Schmidt (7), who fed mice for months on a diet extremely poor in iron, but obtained no fall in the hemoglobin, that the organism possesses great power of conserving its iron and of reutilizing it through some form of intermediary metabolism. When, however, Schmidt withdrew iron from the diet for several generations, the younger generations were extremely anemic and this anemia disappeared upon restoring iron to the diet. As the iron-poor diet led to the disappearance of microchemically demonstrable iron from the liver, but affected to a much slighter degree that of the spleen, Schmidt concluded that the liver is the depot for iron from the food, and that the spleen, on the other hand, is the depot for iron from tissue and erythrocyte catabolism and thus an important factor in the intermediary metabolism of iron.

If the spleen plays this part in iron metabolism, its absence might well interfere with the reutilization of iron by the organism and lead to an increased iron elimination, and this Asher and his coworkers, Grossenbacher and Zimmermann, claim to have demonstrated in dogs. They studied the iron elimination of four puppies from two litters; one from each litter was splenectomized and one from each was kept as a control. The iron estimations were made at intervals of a few weeks, two months, and ten months after splenectomy, and in all their experiments they found an output much higher, often double, in the splenectomized animals as compared with the controls.

Bayer (8) has made some studies on man of the iron elimination following splenectomy for rupture of the spleen or for Banti's disease, and has compared the output for a certain number of days on known diets with that of control cases on the same diets. His results are summarized in the following table.

TABLE I.

Disease.	Age in yrs.	Time after splenectomy.	Output of iron in mg. per kilo per dy.	
			Splenectomized.	Control.
Spleen rupture.....	16	2 wks.	0.22	0.16
			0.15	0.18[2]
Spleen rupture.....	16	3 wks.	0.30	0.22
			0.17	0.08
			0.51	0.50
Banti's disease.....	25	6 mos.	0.19	0.18[2]
Banti's disease.....	19	2 yrs.	0.19	0.18[2]

From these experiments the author concludes that there is an increased output of iron soon after splenectomy, as shown by the second observation in the table, but that later the elimination returns to normal. Bayer's statement that certain of his diets contained 0.24 of a gram of iron per day is probably an error since a diet of the general character that he describes would certainly have a much lower iron content.

METHODS.

In our earlier experiments we studied the iron elimination during four-day periods, but found that these periods led to irregular results. In the work here reported, therefore, we present only observations based on periods of nine or ten days' duration.

The animals were placed in metabolism cages with glass floors and after they had been fed for several days on constant weighed amounts of the diet selected, the rectum was emptied by the use of morphin; iron-free charcoal was added to the next feeding, and the collection of feces was begun from the appearance of the charcoal; at the close of the period the rectum was again emptied with morphin, carmine was added to the next feeding, and the feces were collected until carmine appeared in them. In the earlier experiments

[2] These three figures are merely repetitions of a single control experiment.

the urine also was analyzed, but as only traces of iron, less than 1 per cent. of the total elimination, were found the urine was omitted in our later analyses. To avoid the introduction of extraneous iron, the feces were collected by means of a nickel spatula soon after being passed.

In one group of experiments representing the earlier periods after operation, we have studied the output of iron on the same dogs, both before and after splenectomy, without a change in diet. In another group, representing later periods, we have compared the output of normal control dogs with that of splenectomized dogs of approximately the same weight on corresponding diets.

The analyses were made by the method of Ripper and Schwarzer (9), slightly modified. The feces collected for the entire period are placed in a quartz dish, dried, and ashed dry. The ash is extracted with boiling concentrated hydrochloric acid and filtered, and the residue washed with 20 per cent. hydrochloric acid. The residue and filter paper are re-ashed and the extraction is repeated. This ashing and extraction is continued until the extract ceases to give a positive test with potassium sulphocyanide.

The total filtrate is made up to a known volume and two duplicate portions, containing presumably two to five milligrams of iron, are taken. To each is added one cubic centimeter of hydrogen peroxide (Merck's Blue label), and the solution evaporated to dryness on a water bath. The residue is then redissolved in one cubic centimeter of 20 per cent. hydrochloric acid and twenty cubic centimeters of boiling water are used in four small portions, and then this washing with acid and water is repeated. In the course of the manipulation the entire solution is brought into a 200 cubic centimeter Erlenmeyer flask.

All the specimens to be analyzed at one time having been brought to this stage, a standard is prepared by placing into each of two 200 cubic centimeter Erlenmeyer flasks forty cubic centimeters of a quantitative ferric chloride solution containing about 0.002 of a gram of iron. To each of the flasks, those containing the specimens and the two containing the standard, there are added in rapid succession four grams of potassium iodide; the flasks are then immediately stoppered and placed in a water bath at 60° C. for ten

minutes. At the end of this time the flasks are removed, and to each 100 cubic centimeters of cold water are immediately added and the flask is restoppered.

To each flask in turn is added starch solution, and the contents are titrated with sodium thiosulphate solution, approximately N/250, until disappearance of the blue color, and then they are immediately titrated with weak iodin solution back to the first reappearance of the blue color. In each analysis the thiosulphate solution is freshly prepared and standardized against the two flasks of known ferric chloride solution, and the iodin solution also is freshly prepared and standardized against the thiosulphate solution. The precision of the titration method is found to be greatly enhanced by the titration back with iodin to the first reappearance of the blue color and calculation accordingly of the thiosulphate end point.

In control experiments performed by adding known amounts of iron to one of identical pairs of samples of ash of feces, an error of about 2 per cent. was observed.

The food used in these experiments consisted of casein, cracker meal, lard, and fresh beef heart in proportions designed to give the desired amount of iron. The iron content of the food was determined by analyzing many large portions (each 50 to 400 grams) of the beef heart, cracker, and casein and obtaining average figures for use in calculating the iron content of the diets employed.

RESULTS.

In the accompanying tables are given in detail the final figures obtained in our studies. The experiments are divided into two groups. First, five animals were studied both before and for two weeks after splenectomy, on a constant diet throughout; these are arranged in table II according to the iron content of the diet. Second, a group of six animals (table III), three normal controls and three splenectomized animals, were studied at longer periods after splenectomy; these were of about the same weight and were on diets of the same general character, but varying in the content of iron.

Inspection of table II shows that the iron output of dogs 88 and

35 is unchanged by splenectomy, but that dogs 30, 44, and 79 show some increase. On the other hand, in table III, it will be seen that all three splenectomized dogs exhibit an output of iron as compared with the intake closely comparable with that of the controls. From our studies it would appear therefore that during the first two weeks after splenectomy, some, but not all dogs show a slight increase in the output of iron, but that at 1 month, 9 months, and 20 months after splenectomy we find no indication of an increased iron output. The occasional evanescent and inconstant increase in elimination of iron does not justify the conclusion that the spleen exerts an important influence on iron metabolism. Our

TABLE II.

Dog No.	Average weight.	Duration of periods.	Intake.[3]	Output.[3]		Time after splenectomy.
				Before splenectomy.	After splenectomy.	
88	7,000	10 dys.	0.27	0.67	0.70	4–14 dys.
30	5,340	9 dys.	0.30	0.36	0.55	1–10 dys.
35	7,720	9 dys.	0.64	0.87	0.81	1–10 dys.
44	9,000	9 dys.	1.57	1.89	2.10	1–10 dys.
79	9,000	9 dys.	1.71	1.88	2.21	6–15 dys.

TABLE III.

Dog No.	Controls.				Time after splenectomy.
	Average weight.	Duration of period.	Intake.[4]	Output.[4]	
79	9,000	9 dys.	1.00	1.42	
44	9,000	9 dys.	1.57	1.89	
79	9,000	9 dys.	1.71	1.88	
		Splenectomized.			
83	8,400	10 dys.	1.42	1.39	27–37 dys.
9	8,800	9 dys.	1.35	1.56	9 mos.
51	10,000	9 dys.	1.32	1.42	20 mos.

results are obviously different from those of Asher and his associates, and as a possible explanation of this we would call attention to the extreme shortness of the periods—one to three days—employed

[3] Figures expressing intake and output indicate milligrams of iron per kilo per day.

[4] Figures expressing intake and output indicate milligrams of iron per kilo per day.

by Asher and Grossenbacher, and to their failure to mark in any way the stools. In the studies of output ten months after splenectomy, as given by Asher and Zimmermann, the splenectomized dog in most of the experiments was much larger than the control, so that if the iron output of their dogs be calculated per kilo of body-weight it will be found that the output of the splenectomized animals approaches very closely that of the normal controls, and is in some instances identical. It seems possible that in these studies ten months after splenectomy the increased iron output of the splenectomized animals was due rather to the size of the animal than to the splenectomy, and it is doubtful, therefore, whether the conclusions of Asher and Zimmermann, based on these experiments, are justified.

CONCLUSIONS.

Our studies give evidence of increase in the iron elimination in three of five dogs during a period of two weeks following splenectomy, but not in two other dogs. The occasional increased output of iron may have some relation to the anemia which occurs in the early weeks after splenectomy and which varies in degree in different animals.

No evidence was secured of an increase in the iron output at 1, 9, and 20 months after splenectomy.

From our own studies and from examination of the literature of the subject, we conclude that the spleen does not exercise a constant and important influence upon the iron metabolism of the body.

BIBLIOGRAPHY.

1. Asher, L., and Grossenbacher, H., Beiträge zur Physiologie der Drüsen. Untersuchungen über die Funktion der Milz, *Biochem. Ztschr.*, 1909, xvii, 78. Asher, L., and Zimmermann, R., Beiträge zur Physiologie der Drüsen. Fortgesetzte Beiträge zur Funktion der Milz als Organ des Eisenstoffwechsels, *ibid.*, p. 297.
2. Häusermann, E., Die Assimilation des Eisens, *Ztschr. f. physiol. Chem.*, 1897, xxiii, 555.
3. Abderhalden, E., Die Beziehung des Eisens zur Blutbildung, *Ztschr. f. Biol.*, 1900, xxi, 483.
4. von Voit, C., cited by Hermann, L., Handbuch der Physiologie, Leipzig, 1881, vi, pt. 1, 384.
5. Gottlieb, R., Ueber die Ausscheidungsverhältnisse des Eisens, *Ztschr. f. physiol. Chem.*, 1891, xv, 371.

6. Cetti and Breithaupt, cited by Sherman, H. C., Chemistry of Food and Nutrition, New York, 1911, 247.
7. Schmidt, M. B., Ueber die Organe des Eisenstoffwechsels und die Blutbildung bei Eisenmangel, *Verhandl. d. deutsch. path. Gesellsch.*, 1912, xv, 91.
8. Bayer, R., Untersuchungen über den Eisenstoffwechsel nach der Splenektomie, *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1910, xxi, 335; Weitere Untersuchungen über die Funktionen der Milz, vornehmlich ihre Rolle im Eisenstoffwechsel, mit besonderer Berücksichtigung des Morbus Banti, *idem*, 1913, xxvii, 311.
9. Neuberg, C., Der Harn, sowie die übrigen Ausscheidungen und Körperflüssigkeiten von Mensch und Tier, Berlin, 1911, i, 162.

THE EFFECT OF THE VARIATION IN THE OSMOTIC TENSION AND OF THE DILUTION OF CULTURE MEDIA ON THE CELL PROLIFERATION OF CONNECTIVE TISSUE.*

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PLATES 11 TO 14.

In his fundamental experiments Jacques Loeb has shown the importance of the osmotic tension of water for the growth of marine organisms. Carrel and Burrows,[1] by cultivating for a few days embryonic chick spleen in diluted plasma and in plasma the osmotic tension of which had been modified, attempted to determine what factors could activate the rate of growth of the tissues of higher animals. These experiments were made before Carrel developed the technique which rendered possible the permanent life of connective tissue *in vitro*. Afterwards it became apparent that the modifications of the medium acted differently on tissues proliferating for many generations and tissues maintained for a few days in a condition of survival, and that the conclusions in the article of Carrel and Burrows needed to be modified.

Dr. Carrel gained the impression that it would be necessary, in order to know the influences on cell proliferation of the variation in the osmotic tension and of the dilution of the culture media, to observe for many passages the growth of a tissue in a specific medium. The present experiments were therefore undertaken to determine definitely to what extent strains of connective tissue, kept in a condition of active life, *in vitro*, for many generations, were influenced by the modification of the medium. During the time this work was in progress, an article by Lambert[2] was published on the effect of dilution of plasma on the growth of cells in tissue

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[1] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 562.

[2] Lambert, R. A., *Jour. Exper. Med.*, 1914, xix, 398.

cultures. It was stated that plasma with isotonic solutions causes a more extensive migration in cultures of cells of the actively migratory type, such as those of spleen and bone marrow. Dilution with a limited quantity of distilled water produced the same effect. Less actively motile cells are influenced little or not at all by dilution; also, dilution of the plasma with either isotonic solutions or distilled water is without effect on the cell multiplication. His experiments did not differ in method from those of Carrel and Burrows and are, therefore, open to the same objection.

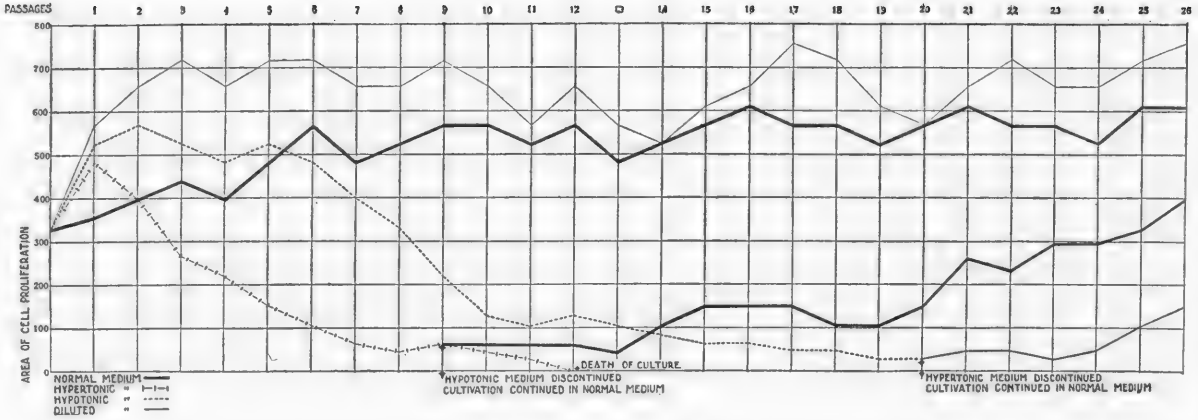
TECHNIQUE.

The strain of connective tissue used as a control was cultivated in a medium, designated normal, composed of one part of freshly prepared embryonic chick extract and of two parts of normal chicken plasma.

Hypotonic medium was prepared by adding two parts of distilled water to three parts of normal plasma and one part of fresh embryonic chick extract.

Preliminary experiments were made to determine the concentration of the salt solution which was added to normal plasma in order to render it sufficiently hypertonic. It was found that a 2.5 per cent. sodium chloride solution was too concentrated. When employed in a culture medium it proved distinctly unfavorable to cell proliferation after one or two passages. A medium containing a 2 per cent. solution of sodium chloride in the same proportion showed that it was possible to keep strains of connective tissue cells in a condition of active life for a number of passages. 1 and 1.5 per cent. solutions were also tried, but the hypertonicity produced thereby was found to be so slight as to approach the conditions which prevailed when normal plasma was diluted with Ringer solution. The hypertonic medium was prepared by adding two parts of a 2 per cent. solution of sodium chloride to three parts of normal chicken plasma and one part of fresh embryonic chick extract.

Diluted medium was prepared by adding two parts of Ringer solution to three parts of normal chicken plasma and one part of fresh embryonic chick extract. Thus, the three modified media, hypotonic, hypertonic, and diluted, contained the same relative amount of normal plasma and fresh embryonic chick extract.



TEXT-FIG. 1. The average area of cell proliferation which developed during forty-eight-hour intervals (one passage) of incubation at 40° C. in cultures of connective tissue which were cultivated in normal, hypertonic, hypotonic, and diluted media, respectively.

Two strains of connective tissue cells were used in these experiments. One strain was derived from a fragment of heart tissue from a chick embryo seven days old, extirpated on January 17, 1912; the other from a fragment of heart tissue from a chick embryo eight days old, isolated on February 18, 1913.

Cultures were made by subdividing fragments of these two strains of connective tissue. The pieces were washed in Ringer solution for three quarters of a minute to one minute and cultivated respectively in normal, hypotonic, hypertonic, and diluted medium. After forty-eight hours' incubation the cultures were washed in Ringer solution as before, transferred into fresh medium, and again incubated. After incubation for one, six, twenty-four, and forty-eight hours, observations were made, and in some instances cultures were fixed and stained. To determine the influence of modified media on the proliferation of connective tissue cells, the growth obtained in a culture, after stated periods in normal medium, was taken as a standard of comparison. The extent of this growth was measured with an ocular micrometer and recorded. The accompanying chart shows, in curves, the relative area of cell proliferation which developed during a passage (forty-eight hours) in cultures which had been grown in modified media. Calculations were made by measuring in the different media the width of the zone of new growth which had developed during a passage. The measurements for all cultures which had been cultivated in the same medium were averaged and the area was computed. The relative density of cell proliferation was determined by observations on stained preparations, and photomicrographs of the preparations were made.

EXPERIMENTS.

Continued cultivation of connective tissue in hypertonic medium produced the following results: When a culture was transferred to hypertonic medium from a normal medium in which the width of the area of cell proliferation after forty-eight hours' incubation was equal to seven divisions of the ocular micrometer, observations showed, after one hour's incubation, no evidence of cell proliferation; after six hours' incubation a few elongated cells were observed spreading from the periphery of the central fragment into the

medium; after twenty-four hours the area of cell proliferation measured, on the average, six and a half divisions, slightly more than the area of cell proliferation in the control, which measured six divisions. Stained preparations at this stage of incubation showed the area to be denser than the control. After forty-eight hours' incubation the zone of proliferating cells measured nine divisions, and in the control seven and a half divisions. The density was greater in comparison with the normal control and the contrast could be observed in the living culture. Examination of the cells in these cultures showed the presence of many refractile globules of varying size in the cytoplasm of the cell. In the control, cells showed small refractile globules, few in number and fairly uniform in size. Preparations stained with Sudan III and hematoxylin showed these refractile globules to be fatty substances which had accumulated in the cell cytoplasm. A third passage into hypertonic medium after forty-eight hours' incubation produced a less extensive, though quite as dense an area of cell proliferation as the control. The zone measured six divisions, and the control nine and a half. The amount of fat present in the cytoplasm of the cell was observed to be apparently as great as in the previous passage. There was no increased amount of fat present in the control over that present in the new cells which developed in the previous passage. A fourth and fifth passage, after forty-eight hours' incubation, respectively, showed a decided decrease in extent of new growth. The area of cell proliferation appeared to be as dense as the area of cell proliferation in the control, but the central portion of the culture had become a thick and opaque mass. The cells were observed to be filled with large fat granules. After the sixth and seventh passages the extent of cell proliferation was decidedly less than the control, measuring two and a half divisions, whereas the extent of growth in the control measured nine and a half. The central portion of the cultures had contracted into a very dense, spherical, opaque mass. Further passages into hypertonic medium resulted in a rapid degeneration of the culture, with central necrosis and death after the eleventh or twelfth passage. In some instances tissues which had been cultivated for nine passages (eighteen days) in hypertonic medium were revived by again cultivating them in

normal medium, and after seventeen passages (thirty-four days) the extent of cell proliferation was approaching normal. In the first few days during which a culture was cultivated in hypertonic medium the area of new growth was more extensive and apparently more dense than in the control. After that time this area decreased rapidly and eventually the culture died.

Cultivation of connective tissue in hypotonic plasma after one hour's incubation showed new cells budding out from the peripheral portions of the central fragment. The same condition was observed in the control. After six hours' incubation the area of cell proliferation was about the same as the control, but in twenty-four hours the zone of proliferating cells was more extensive than in the control, measuring about eight and a half divisions in width, whereas the width of the area in the control measured slightly over seven divisions. After forty-eight hours' incubation the increase in extent of cell proliferation was decidedly greater than the control, measuring about nine and a half divisions, as compared with seven and a half divisions in the control. The density of growth, however, was less than the control. Stained preparations showed fewer planes in which cell proliferation had taken place. After the second and third passages, respectively, the extent of growth was still observed to be greater (about nine and a half divisions) than the control (about seven and a half divisions). The accumulation of fat globules in the cytoplasm of the cells was practically the same as the control. After the fourth and fifth passages the area of proliferating cells was slightly greater than the control, but the relative density was less in the former. After the sixth and seventh passages the extent of cell proliferation decreased and was observed to be less than the control. The cells showed an increase in fat accumulation over the control and the cells appeared loosely joined, forming a large meshed network of interlacing cells. Continued cultivation in hypotonic medium up to twenty passages (forty days) showed a gradual decrease in the extent of growth. The central fragment became dense and opaque and large fat globules were observed in the cytoplasm of the cells. After twenty passages in hypotonic medium some cultures were again cultivated in normal medium. They usually recovered after five or six passages. During a period last-

ing about ten days, a culture cultivated in hypotonic medium showed that the area of cell proliferation was more extensive though less dense than the normal control. After that time the extent of growth decreased gradually and finally cell proliferation became sluggish. It was possible to revive again a culture at this stage.

The cultivation of cultures of connective tissue in diluted medium after one hour's incubation showed a number of new cells growing out into the medium from the periphery of the central fragment. In six hours an appreciable crown of new cells encircled the central portion of tissue, and after twenty-four hours the area of new growth was more extensive (about eight and a half divisions in width) than the control (about seven divisions in width). After forty-eight hours it was observed that the area of cell proliferation which had developed was decidedly more extensive (width of area eleven divisions) than the control (width of area seven and a half). Stained preparations showed this area to be less dense than the control, but there was no increase in the amount of fat globules present in the cytoplasm of the cells. A second, third, and fourth passage showed an increase in area over the control and many passages thereafter (twenty-two passages, forty-four days) showed that this increase in area of cell proliferation over the normal was fairly constant. This point is shown in the text-figure 1. The fluctuations of growth which were observed in the control were also observed at the same time in the experiment. When the extent of cell proliferation was less extensive in normal medium than in the previous passage, it was found that there was a relative decrease, on most occasions, in the extent of cell proliferation in diluted medium. The stained preparations of cultures cultivated in diluted medium for many passages showed an extensive, loosely meshed network of elongated cells. The control showed a more densely packed mass of elongated cells, but the area of cell proliferation was less. Figure 1 represents an entire culture of connective tissue forty-eight hours after passage into normal medium, which had been cultivated for about two hundred passages in this medium. Figure 2 shows an entire culture of connective tissue forty-eight hours after the last passage into diluted medium. The last passage was the twenty-fifth consecutive passage into diluted medium. Figure

3 is a higher magnification of part of a control culture after forty-eight hours' incubation. Figure 4 shows a portion of the area of cell proliferation which developed in a culture in forty-eight hours in diluted medium, after twenty-two passages into the same medium. The relative increase in the extent of cell proliferation in diluted medium, over the control, remained fairly constant through the total number of passages, although no actual increase in mass was observed (text-figure 1).

When connective tissue was cultivated in hypertonic medium for one passage and then in hypotonic medium, observations showed no marked differences in cell proliferation, but alternate passages from hypertonic to hypotonic medium for three or four passages caused death of the culture. Alternate passages from hypertonic medium into diluted medium proved unfavorable after three or four passages, and usually resulted in death of the culture after five generations. Alternate passages from hypertonic medium into normal medium proved unfavorable after five passages, and when continued for ten passages the culture died. Alternate passages from hypotonic into normal medium were continued for ten generations. A gradual decrease in the extent and activity of cell proliferation was observed and this treatment proved unfavorable for growth in the culture. Observations showed that alternate passages from normal medium into diluted medium did not appear to affect the dynamic condition of the culture, although, relatively, there appeared to be a denser area of cell proliferation in normal medium and a less dense but more extensive area of growth in diluted medium. Alternate passages of connective tissue into modified media in most instances produced unfavorable results.

SUMMARY.

For the first few days of cultivation of connective tissue in hypertonic, hypotonic, and diluted medium, cell proliferation was stimulated. The first outgrowths of new cells in the modified media did not occur sooner than in normal medium. In hypertonic medium the density of the area of cell proliferation appeared to be greater than the control, but in hypotonic or diluted medium there seemed to be no increase in actual mass over the control. These observa-

tions confirm the conclusions of Carrel and Burrows, as well as those of Lambert.

Subsequent to the first few days of cultivation in hypertonic medium the area of cell proliferation decreased and in a short time conditions developed which were unfavorable to growth, and finally resulted in death of the culture, unless it revived before this stage. Hypotonic medium after about ten days no longer caused more extensive areas of proliferating cells; but instead, the extent of new growth gradually decreased, and the culture merely remained alive unless revived. In diluted medium the extent of the area of cell proliferation remained greater with no actual increase in mass. The area of cell proliferation which is observed during the first few days in a culture of fresh tissue recently extirpated does not indicate the actual influence of modified media. It was only after continued cultivation of strains of connective tissue in these modified media that their influence on cell proliferation was determined.

CONCLUSION.

An increase or decrease in the osmotic tension of the culture medium at first stimulates cell proliferation, but eventually retards it and proves to be unfavorable to growth. Dilution of the medium without change of the osmotic tension produces a more extensive zone of cell proliferation but no increase in the actual mass of newly formed tissue. A culture of connective tissue, which has been growing under unfavorable conditions, due to changing the osmotic tension of the medium in which it has been cultivated, is capable of being revived.

EXPLANATION OF PLATES.

PLATE 11.

FIG. 1. The extent of growth obtained after forty-eight hours' incubation in normal medium. This culture had been cultivated in normal medium for about two hundred passages. Control for culture in figure 2. Stained preparation; low power.

PLATE 12.

FIG. 2. The extent of growth obtained after forty-eight hours' incubation in diluted medium. This culture had been cultivated in diluted medium for twenty-five passages (fifty days). Stained preparation; same magnification as figure 1.

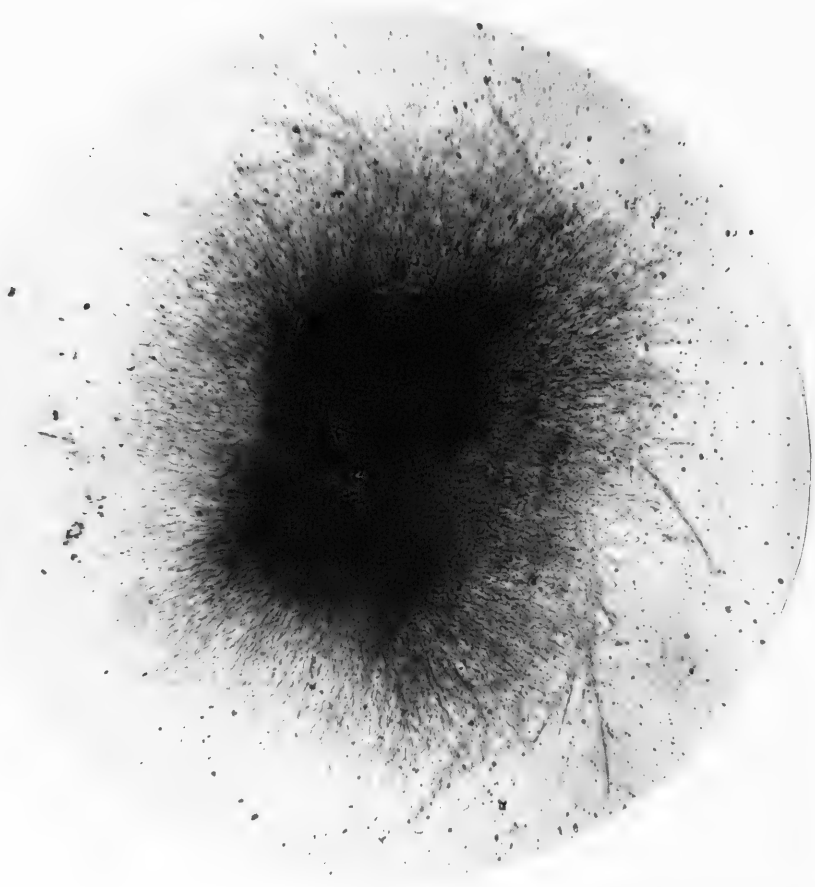


FIG. 1.

(Ebeling: Cell Proliferation of Connective Tissue

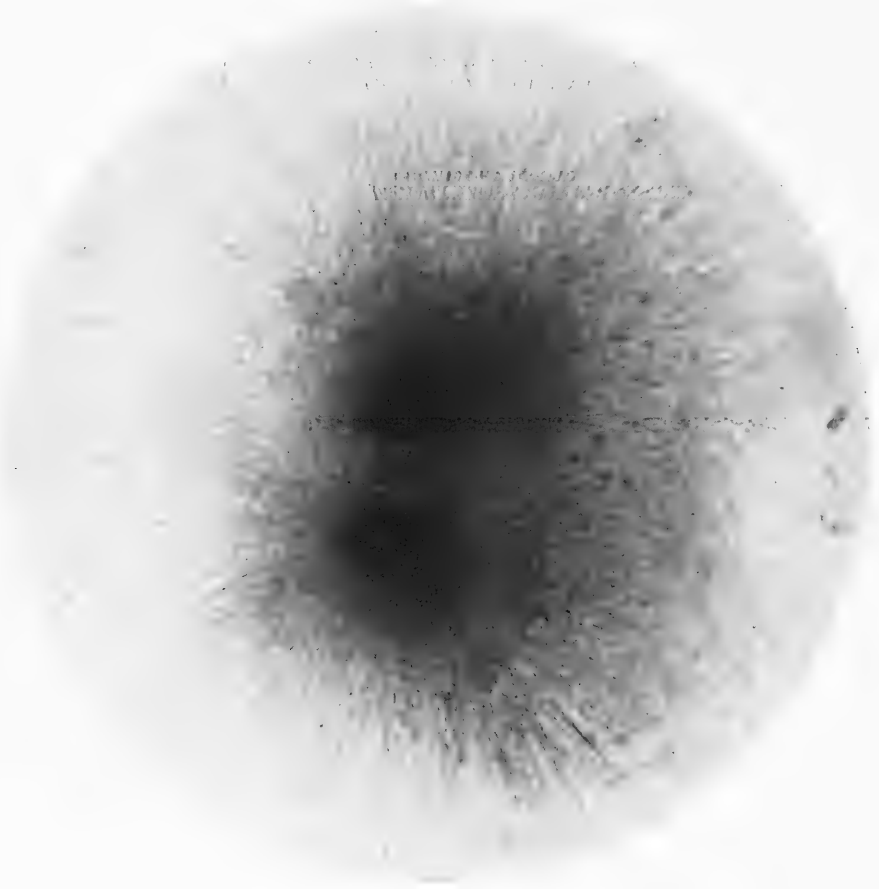


FIG. 2.

(Ebeling: Cell Proliferation of Connective Tissue.)

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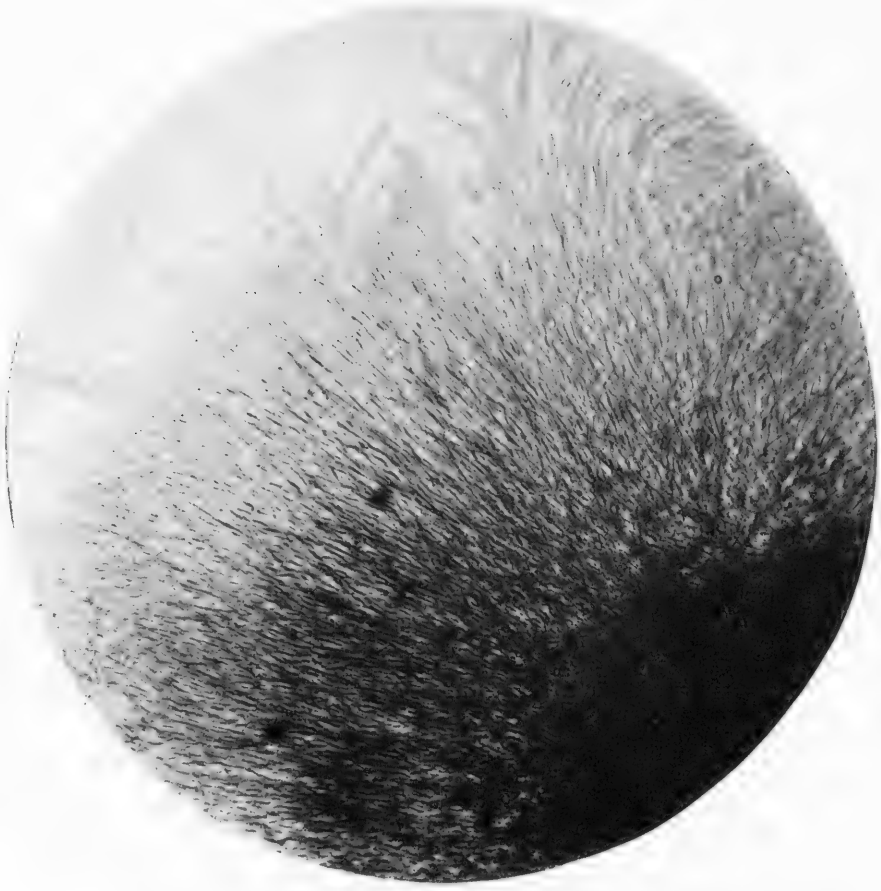

FIG. 3.

(Ebeling: Cell Proliferation of Connective Tissue.)

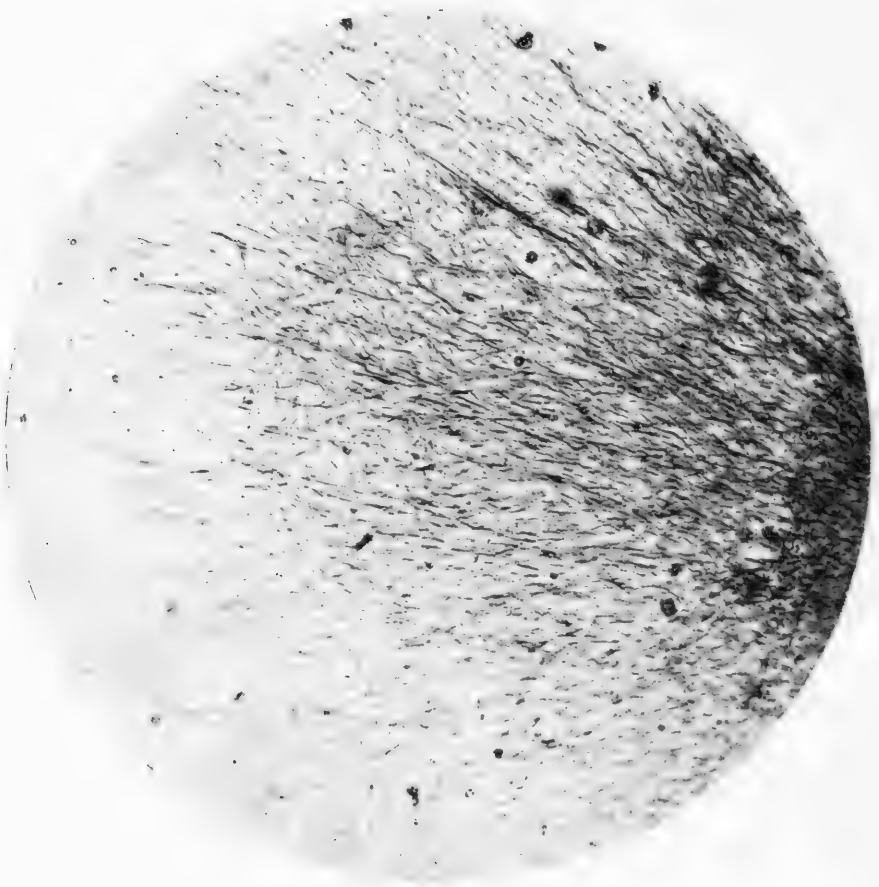

FIG. 4.

(Ebeling: Cell Proliferation of Connective Tissue.)

PLATE 13.

FIG. 3. The extent of the area of cell proliferation which developed after forty-eight hours' incubation in a normal control. Stained preparation; higher power.

PLATE 14.

FIG. 4. The area of cell proliferation which developed after forty-eight hours' incubation in diluted plasma. This culture had been cultivated in the same medium for twenty-three generations (forty-six days). Stained preparation; same magnification as figure 3.

THE CULTIVATION OF HUMAN SARCOMATOUS TISSUE IN VITRO.*

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(From the Laboratories of the New York Lying-In Hospital and of The Rockefeller Institute for Medical Research.)

Plates 15 to 17.

The first attempt to cultivate human malignant tumor *in vitro* was made in 1911 by Carrel and Burrows.[1] Small fragments of tumor were cultivated in normal human plasma and incubated. It was observed in some cases that after a few days the fragments were surrounded by many cells; but generally liquefaction of the medium occurred. The tissues were kept in a condition of survival for a few days, but no real cultures were obtained.

Lately it became possible to keep human fetal tissue, derived from fresh cadavers, in a condition of independent life for several generations,[2] and we therefore attempted to cultivate human sarcomatous tissue in the same manner.

TECHNIQUE.

The medium employed in these experiments was composed of equal parts of normal human plasma and Ringer solution and varying quantities of extract.

The extract was prepared by cutting tissues obtained from fresh fetal cadavers into small pieces, and adding an equal quantity of Ringer solution. After forty-eight hours in cold storage the substance was centrifuged and the supernatant fluid pipetted off. This fluid was used as extract in the making of cultures.

The tissues employed were obtained from recently excised sarcomatous growths,[3] and cultures were made about one and a half hours

* Received for publication, June 3, 1914.

[1] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 387.

[2] Losee, J. R., and Ebeling, A. H., *Jour. Exper. Med.*, 1914, xix, 593.

[3] The sarcomatous growths were obtained by Dr. Carrel, through the courtesy of Dr. W. B. Coley, from some of his cases at the General Memorial Hospital, New York. Immediately after excision the tissues were carried to the Laboratories of the New York Lying-In Hospital.

after excision. The primitive cultures were made by putting small, thin fragments of this tissue into the medium. After coagulation the cultures were immediately placed in the incubator and incubated at 38° C. for 24, 48, and 72 hours, the time of passage into fresh medium being governed by conditions which developed in the culture. Before the fragments in cultures were transferred into fresh medium they were washed in Ringer solution for about one minute.

EXPERIMENTS.

Experiment 1, Series 1, Cultures 1, 2, 3, and 4.—Fragments from the periphery of an osteosarcoma[4] were cultivated in equal parts of human plasma and Ringer solution, to which one fourth part of extract was added. The cultures were made about one hour and a half after excision of the growth and incubated.

After 1, 6, and 18 hours' incubation there was no evidence of cell proliferation in any of the cultures. In twenty-four hours there was still no growth to be observed and the medium had become liquefied around the fragments. The cultures were therefore washed and changed into fresh medium (first passage), to which one half part of extract was added. After twenty-four hours' incubation, growth was found to be present in culture 3, but no growth was observed in cultures 1, 2, and 4, and in forty-eight hours there was still no evidence of cell proliferation in these cultures; the medium had become liquefied, and they were therefore discarded.

Culture 3 was cultivated in the same medium (second passage), and in forty-eight hours growth was apparent. This culture was transferred into fresh medium for twelve passages, during which time (twenty-one days) growth was observed after each transfer into fresh medium. The culture was stained and photographed (figure 1).

Series 2, Cultures 1, 2, 3, and 4.—Fragments from the peripheral area of the same growth were cultivated in the same manner as series 1.

After 1, 6, and 18 hours' incubation there was no evidence of cell proliferation. The medium was still in good condition. In twenty-four hours no growth was observed, and the medium about the

[4] Pathological diagnosis: large round cell sarcoma.

pieces in cultures 1, 2, and 3 had become liquefied. Culture 4 was allowed to remain in the incubator. It was examined after forty-eight and seventy-two hours, but there was no evidence of cell proliferation, although the medium was still in good condition. The culture was discarded. After twenty-four hours cultures 1, 2, and 3 were changed into fresh medium (first passage), to which one half part of extract was added.

Culture 1 after twenty-four hours showed no growth, and the medium was completely liquefied. It was changed into fresh medium (second passage), the same proportion of extract being added as in the previous passage. In twenty-four hours a few scattered cells were observed, but after forty-eight and seventy-two hours there was no further increase in the extent of cell proliferation. The culture was discarded.

Culture 2 (first passage) after twenty-four hours' incubation showed an area of cell proliferation, with no liquefaction of the medium. In seventy-two hours a good growth had developed. The medium was slightly liquefied. It was then changed into fresh medium (second passage), to which one fourth part of extract was added. After twenty-four hours growth had developed, and in forty-eight hours the area of cell proliferation was more extensive, but liquefaction of the medium had developed. The culture was changed into fresh medium (third passage) with the same proportion of extract added as in the previous passage. After twenty-four hours a few cells were observed in the medium surrounding the central fragment. In forty-eight hours there was no increase in the extent of growth and the medium had liquefied. The culture was again transferred into fresh medium (fourth passage), one part of extract being added. In twenty-four hours the medium had become liquefied and no growth was observed. The culture was changed (fifth passage) into fresh medium, the same proportion of extract being added as in the previous passage. In twenty-four hours growth had developed, and in forty-eight hours it was more extensive, but the medium was almost completely liquefied. The culture was transferred (sixth passage) into fresh medium in the same manner as in the previous passage. After twenty-four hours cell proliferation was observed, but small colonies of bacteria had also developed. The infection was general and the culture was discarded.

Culture 3 (first passage), after twenty-four hours' incubation, showed a few scattered cells which had grown out from the original fragment. In forty-eight hours the growth of new cells had increased, but the medium was slightly liquefied. The culture was then treated (second passage) in the same manner as in the previous passage. After twenty-four hours good growth had developed and the medium was in good condition. After forty-eight hours the growth was more extensive, but after seventy-two hours there was no further increase and the medium was slightly liquefied. The third passage into fresh medium was then made, the proportion of extract being increased to one part. After twenty-four hours' incubation the growth had developed and the medium was in good condition. In forty-eight hours the culture was growing actively and the medium had become slightly liquefied. The culture was subsequently changed into fresh medium for twenty-one more passages. The extent of growth fluctuated and gradually decreased. In the fifteenth passage the culture was divided and two cultures were made; one of the cultures (figure 2) was fixed after forty-eight hours' incubation. After the twenty-fourth passage cell proliferation stopped. This culture was transferred twenty-five times, during a period of fifty-one days. The entire history is given in table I, which shows the passage, treatment, and observations that were made on culture 3.

Experiment 2.—A series of cultures, Nos. 1, 2, 3, and 4, was made from fragments of the periphery of a large round cell sarcoma,[5] and cultivated in equal parts of plasma and Ringer solution. After twenty-four hours there was no evidence of growth in any of the cultures and the medium was in good condition. In forty-eight hours there was evidence of cell proliferation in all cultures. After seventy-two hours the area of cell proliferation had increased, but the medium in cultures 3 and 4 had become liquefied. Cultures 1 and 2 were stained.

Cultures 3 and 4 were changed into fresh medium (first passage), and one part of extract was added to the medium. Both cultures developed good growth in forty-eight hours with no liquefaction of the medium. They were again transferred into fresh medium

[5] Pathological diagnosis: large round cell sarcoma.

TABLE I.

Passage.	Date (1913).	Treatment of culture.	Observations.
Experiment 206–3	Nov. 18	Culture of a fragment of the peripheral part of an osteo-sarcoma, cultivated in 1 part of human plasma, 1 part of Ringer solution, and $\frac{1}{4}$ part of extract	Nov. 19. Medium liquefied; no growth.
1	Nov. 19	Washed in Ringer solution for 1 minute, cultivated in 1 part of human plasma, 1 part of Ringer solution, and $\frac{1}{2}$ part of extract	Nov. 20. A few scattered cells; medium in good condition. Nov. 21. Good growth; medium slightly liquefied.
2	Nov. 21	Treated in the same manner as in previous passage	Nov. 22. Good growth; medium in good condition. Nov. 23. Growth more extensive; medium in good condition. Nov. 24. No increase in extent of growth; medium slightly liquefied.
3	Nov. 24	Washed in Ringer solution for 1 minute, cultivated in 1 part of human plasma, 1 part of Ringer solution, and 1 part of extract	Nov. 25. Good growth; medium in good condition. Nov. 26. Growing actively; medium slightly liquefied.
4	Nov. 26	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 27. Good growth; medium in good condition. Nov. 28. Growth more extensive; medium in good condition. Nov. 29. No increase in extent of growth; medium liquefied.
5	Nov. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 30. A few cells; medium in good condition. Dec. 1. A few cells; medium slightly liquefied.
6	Dec. 1	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 2. A few scattered cells; medium in good condition. Dec. 3. Good growth; medium in good condition.
7	Dec. 3	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 4. Good growth; medium in good condition. Dec. 5. Very good growth; medium in good condition.
8	Dec. 5	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 6. Good growth; medium slightly liquefied.
9	Dec. 6	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 7. Good growth; medium in good condition. Dec. 8. More extensive growth; medium liquefied.
10	Dec. 8	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 9. Good growth; medium partially liquefied. Dec. 10. No increase in amount of growth; medium liquefied.

TABLE I.—*Continued.*

Passage.	Date (1913).	Treatment of culture.	Observations.
11	Dec. 10	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 11. Good growth; medium in good condition. Dec. 12. Growth increased; medium in good condition.
12	Dec. 12	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 14. Good growth; medium in good condition. Dec. 15. Very good growth; medium in good condition.
13	Dec. 15	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 16. Growing; medium in good condition. Dec. 17. Very good growth; medium slightly liquefied.
14	Dec. 17	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 18. Good growth; medium in good condition. Dec. 19. Extensive growth; medium in good condition.
15	Dec. 19	Washed in Ringer solution for 1 minute, divided into 2 parts, and cultivated in the same medium (cultures 1 and 2)	Dec. 20. Good growth in cultures 1 and 2; medium in good condition. Dec. 21. Good growth; medium in good condition, No. 2 fixed and photographed. Dec. 22. Good growth; medium in good condition.
16	Dec. 22	Washed in Ringer solution for 1 minute, divided into 2 parts, and cultivated in the same medium	Dec. 23. Growing; medium in good condition. Dec. 24. Growing; medium partially liquefied.
17	Dec. 24	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 26. Growing; medium in good condition.
18	Dec. 26	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 27. A few cells; medium in good condition. Dec. 28. Growing; medium in good condition. Dec. 29. Growing; medium in good condition.
19	Dec. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 30. A few cells; medium in good condition. Dec. 31. Growing; medium in good condition.
20	Dec. 31	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 2. Growing; medium in good condition.
21	(1914) Jan. 2	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 3. No growth; medium in good condition. Jan. 4. A few cells.
22	Jan. 4	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 5. A few cells; medium in good condition. Jan. 6. Slow growth; medium slightly liquefied.
23	Jan. 6	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 7. No growth. Jan. 8. A few scattered cells; medium in good condition. Jan. 9. Growing slowly; medium slightly liquefied.

TABLE I.—*Concluded.*

Passage.	Date (1914).	Treatment of culture.	Observations.
24	Jan. 9	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 10. Slight growth; medium in good condition. Jan. 11. No increase in growth; medium in good condition.
25	Jan. 11	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 12. No growth. Jan. 13. No growth. Jan. 14. Discarded.

(second passage). Culture 4 showed a few proliferating cells after seventy-two hours' incubation, and the medium was in good condition. The culture was discarded. After twenty-four hours' incubation in the second passage culture 3 showed growth with no liquefaction. In forty-eight and seventy-two hours growth had increased, but the medium was partially liquefied. The culture was transferred into fresh medium (third passage) and showed a few cells which had spread out into the medium from the central fragment. After seventy-two hours there was no increase in the number of cells, and the medium had liquefied. The culture was transferred into fresh medium (fourth passage), and after forty-eight hours good growth was observed. The medium was in good condition. The fifth passage into fresh medium was made in the same manner as in the previous passage. After twenty-four hours the medium was totally liquefied and no growth was observed. The fragment was transferred to fresh medium, but after 24, 48, and 72 hours no growth developed. The culture was discarded.

RESULTS.

Two experiments were made in which fragments from human sarcomatous tissue were cultivated. It was possible to keep cultures of such tissue in a condition of active life *in vitro* for several generations.

During the first twenty-four hours of incubation there was usually no evidence of cell proliferation, and slight liquefaction around the primitive fragments. When no liquefaction occurred, growth of new cells manifested itself after forty-eight hours. Twenty-four hours after passage into fresh medium (first passage), cell proliferation was observed in those cultures which showed no evidence of

growth when first cultivated. In comparison with human connective tissue, the rate of growth was practically the same in the beginning, but a gradual decrease in the activity and extent of cell proliferation was observed as the length of time increased during which the culture was carried through successive passages. Microscopic examination of the first outgrowth of cells showed the presence of large, round, as well as elongated and ramified cells. In subsequent passages the round cells were no longer to be identified, and the elongated, ramified variety only were observed. The morphological characteristics of these cells did not appear to differ from the cells present in cultures of normal human connective tissue. Preparations stained with Giemsa stain showed the large round cells as having a densely stained cytoplasm with from one to two nuclei and a regular outline. The elongated and ramified varieties showed no difference in comparison with those present in cultures of human connective tissue, with the exception that no mitotic figures were observed. Figure 3 shows a few of the peripheral cells in a culture of sarcomatous tissue which had been carried through twelve passages.

One culture was stained which was growing actively in its twelfth passage (twenty-one days). This culture is shown in figure 1, the area of cell proliferation being that which developed in the twelfth passage during forty-eight hours' incubation. One other culture was carried through for twenty-four passages, that is, fifty-two days. It was possible to divide this culture in its fifteenth passage, making two, and after forty-eight hours' incubation one of these cultures was fixed. Figure 2 shows almost the entire culture.

Sarcomatous tissue grew as well during a few days as normal connective tissue. Afterwards the rate of growth became less rapid and the tissue could not be kept alive for more than fifty-two days, while normal human tissue could be kept for sixty-eight days.

These differences may be due to technical factors, but they may also be the result of the nature of the tissue itself. In his attempts at keeping Rous sarcoma in a condition of permanent life *in vitro*, Carrel[6] observed that after a few generations the rate of growth became less rapid than the rate of growth of connective tissue. In

[6] Carrel, A., *Jour. Exper. Med.*, 1912, xv, 516.

other experiments with rat sarcoma and normal rat connective tissue, cultivated in guinea pig plasma, Carrel also observed the same differences. The writers observed the same phenomena when rat sarcoma and normal heart tissue of the rat were cultivated in chicken plasma.

The results obtained show that it is possible to cultivate *in vitro* fragments of human sarcomatous tissue for several generations, and that the method employed may prove of value in the study of the growth of human malignant tumor.

EXPLANATION OF PLATES.

PLATE 15.

FIG. 1. 12th passage of human sarcomatous tissue. The preparation shows the area of cell proliferation that developed during forty-eight hours' incubation. Giemsa stain.

PLATE 16.

FIG. 2. 15th passage of human sarcomatous tissue. The photograph shows the growth obtained forty-eight hours after passage into fresh medium. Fixed specimen.

PLATE 17.

FIG. 3. High power magnification of some of the peripheral cells present in the same preparation shown in figure 1.

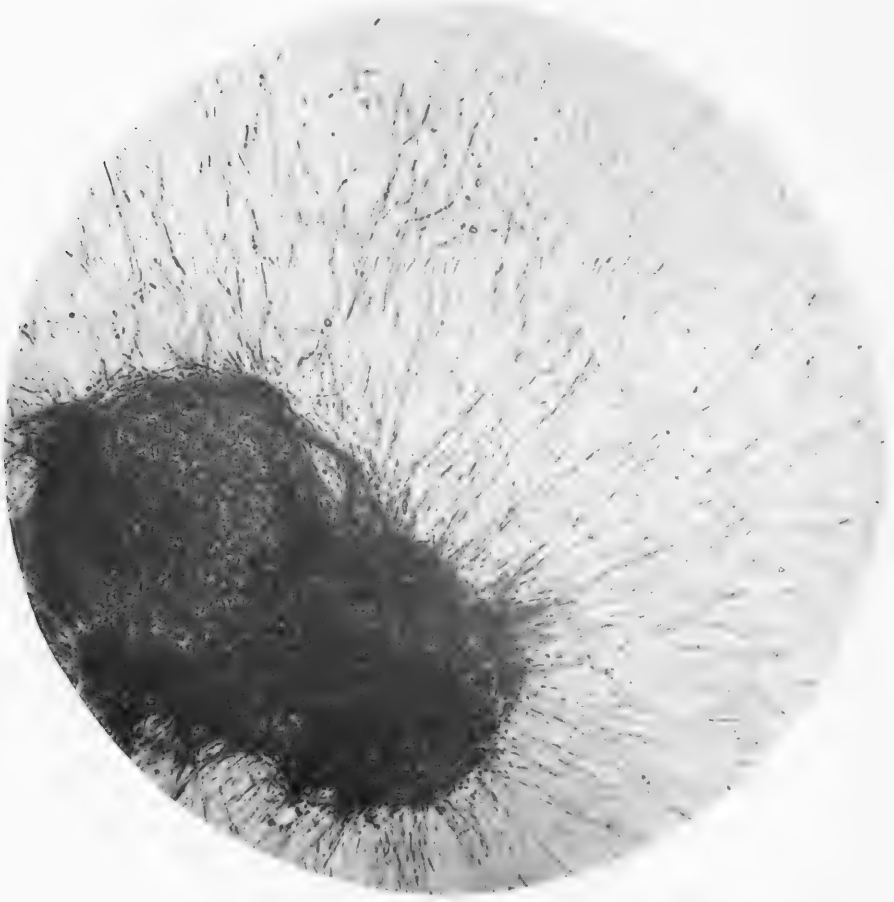

FIG. 1.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro*.)

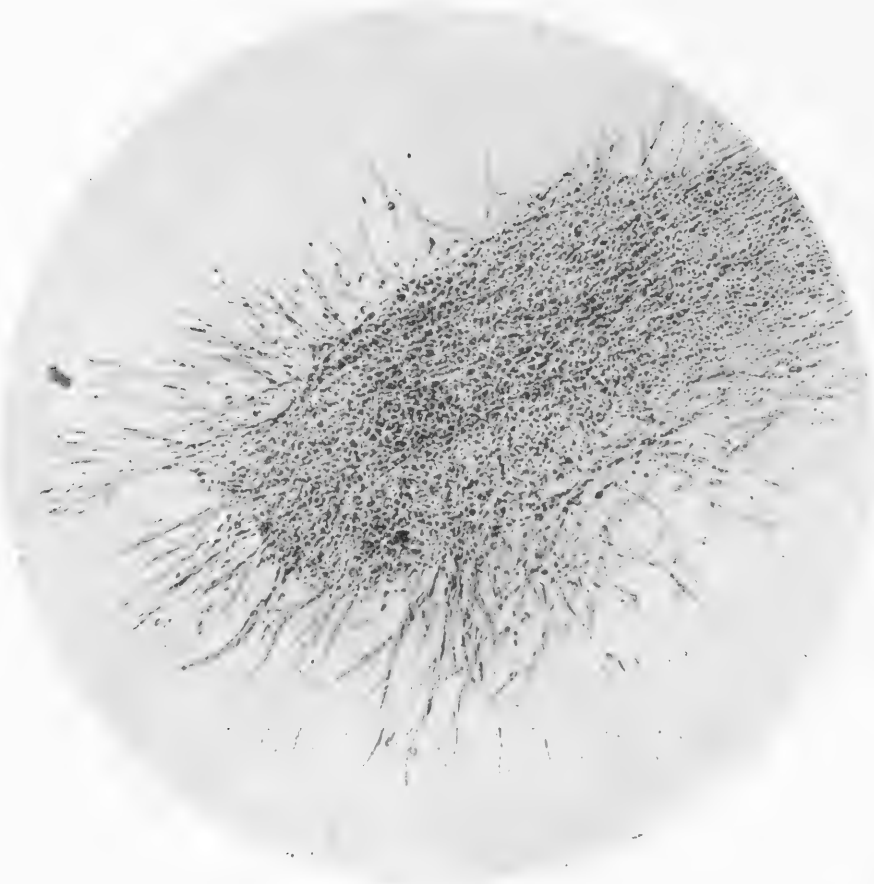

FIG. 2.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro*.)

FIG. 3.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro*.)

THE REMOVAL OF CALCIUM FROM THE BLOOD BY DIALYSIS IN THE STUDY OF TETANY.*

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On the appearance of the paper by Abel, Rowntree, and Turner[1] describing a method by which diffusible substances might be removed from the circulating blood by dialysis, it occurred to us that we might attack the question of the relation of calcium salts to tetany in a new way.

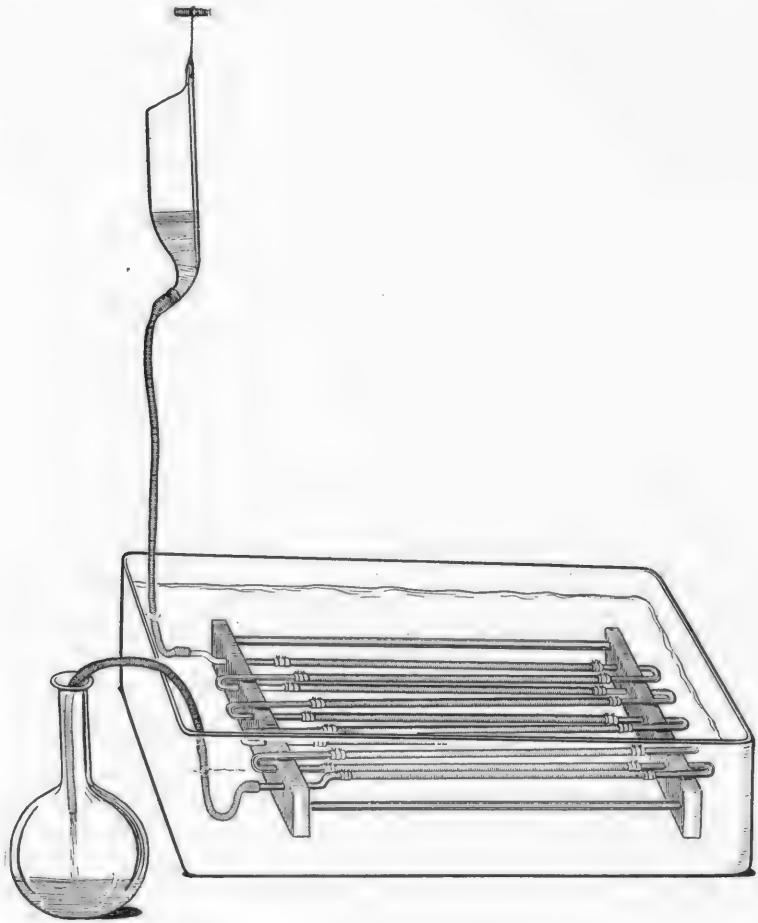
At first we thought that we might be able to produce tetany by mechanically removing calcium from the blood by allowing it to circulate through celloidin tubes immersed in an isotonic solution containing no calcium. We therefore constructed a machine somewhat like Abel's, and made a number of experiments with this object in view, using a fluid put together according to Abderhalden's analysis of the inorganic constituents of the dog's blood, but without calcium. We varied this fluid by substituting phosphates for chlorides, with the idea that by removing chlorides also we might still further reduce the proportion of soluble calcium. We used defibrinated blood instead of salt solution in starting the current, and finally defibrinated all the animal's blood. The results were practically negative, and we abandoned the method because it seemed that we could attain our end better in another way without exposing the animal to an operation which must be prolonged over many hours.

We adopted a method which consisted in passing a quantity of defibrinated blood from another dog through a modified dialyzing apparatus (text-figure 1) for many hours until, as we could ascer-

* Received for publication, June 2, 1914.

1 Abel, J. J., Rowntree, L. G., and Turner, B. B., *Jour. Pharmacol. and Exper. Therap.*, 1914, v, 275.

tain by chemical analysis, the amount of calcium was greatly reduced and had passed over into the dialyzing fluid. It was then



TEXT-FIG. 1. Dialysis apparatus of celloidin tubes in series.

proposed to run the blood into the veins of a normal animal after an equal amount had been removed from the carotid artery, but even before this was done, it was thought best to ascertain the effect of

the dialyzed blood upon the nerves of an isolated extremity by perfusion, according to the method previously employed[2] in studying the blood of animals in tetany. A number of experiments made in this way gave the following results.

Dog 1,420.—Normal extremity. Perfusion started at 3.50 P. M. with blood dialyzed against calcium-free fluid.

	2.00	3.30	3.50	4.00	4.10	4.20	4.40	4.50
KC[3]	0.2	0.05	Perfusion started	0.05	0.05	0.05	0.1	0.2
KO	—[4]	—		0.6	1.8	1.2	1.6	2.4
AC	0.5	1.0		0.2	0.5	0.6	0.4	1.2
AO	1.4	0.4		0.5	0.5	0.6	0.4	1.2

Blood used in perfusion contained calcium per 1,000 c.c. as follows:

Before dialysis.	After dialysis.	After perfusion.
0.085	0.0465	0.080

Dog 1,421.—Normal isolated extremity. Perfusion started at 4.15 P. M. in the same way.

	3.50	4.00	4.15	4.20	4.30	4.45	5.00	5.15	5.30
KC	0.4	0.6	Perfusion	0.2	0.4	0.4	0.4	0.4	0.4
KO	—	—		1.6	2.8	1.8	1.8	2.6	1.6
AC	1.0	1.6		1.2	1.0	1.0	1.2	1.0	1.0
AO	1.2	2.0		1.2	1.0	1.0	1.0	0.8	1.0

Dog 1,427.—Normal leg perfused first with normal then with dialyzed blood.

	8.45	9.00	9.30	9.45	9.50	10.00	10.10	10.15	10.20	10.30	10.40	10.50	11.00	11.10	11.20	11.30	11.35	11.50
KC	0.3	0.4	Perfusion complete Normal blood	0.6	0.8	0.8	1.0	1.0	0.6	0.6	0.6	0.6	0.8	0.8	1.0	1.0	2.0	1.2
KO	—	—		—	—	—	—	—	4.4	3.6	4.0	3.4	3.4	—	—	—	—	—
AC	1.6	1.6		2.4	2.4	2.4	2.8	2.4	2.0	2.0	2.0	2.0	2.2	2.2	2.4	3.4	3.4	4.0
AO	1.8	2.4		—	4.6	4.6	3.2	2.6	2.0	2.0	2.0	2.0	2.0	1.4	2.4	2.2	2.6	4.0

Dialyzed blood | Normal blood

[2] MacCallum, W. G., *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1913, xxv, 941.

[3] KC = kathode closing; AO = anode opening, etc.

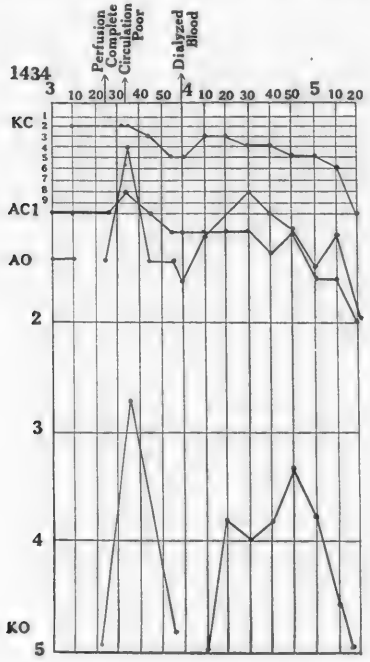
[4] In all the tables — indicates negative up to 5 milliamperes.

It will be seen from these tables that the results of perfusion with dialyzed blood were not especially striking, nor do they convince one that the alterations in excitability of the nerves are strictly due to the withdrawal of calcium. From long previous experience, although it is known that a rise in excitability of a brief and temporary nature can come about from inadequacy of the circulation and that the great fall at the end of each curve is doubtless due to this or to some direct injury of other kinds, still it is also known that with a good circulation with normal blood the excitability will remain almost constant. These curves were especially inconclusive in that while a visible contraction could be elicited with weak currents, strong shocks gave only about the same muscular jerk. Instead of a brisk sharp contraction the foot moved lazily, often twisting in a peculiar way, and after the perfusion with dialyzed blood had gone on for only a short time all peripheral contractions disappeared and the shock produced only jerking of the thigh muscles behind the electrode. These are inevitable signs of approaching death of the nerve, impressing us rather as the consequences of some sort of poisoning, and in every case the dialyzed blood was quickly exchanged for normal blood, sometimes with a return of the excitability.

Discouraged by these results, we determined to add the normal amount of calcium to our dialyzing fluid, which at this time had the following composition:

Sodium chloride	60.0
Disodium phosphate	28.5
Potassium chloride	4.0
Magnesium chloride	2.87
Sodium bicarbonate	5.0
Dextrose	10.9
Water	10,000

It became apparent at once, as we might have foreseen, that the calcium is precipitated by the phosphates present. Phosphates were therefore left out and calcium was added. After dialysis with this fluid, perfusion of the leg gave almost the curve which one would expect with normal blood.



TEXT-FIG. 2. Perfusion of leg with blood dialyzed against fluid containing all inorganic constituents of blood including calcium.

Dog 1,434.—(Text-figure 2.) Normal leg perfused with blood dialyzed against a fluid containing calcium but no phosphates.

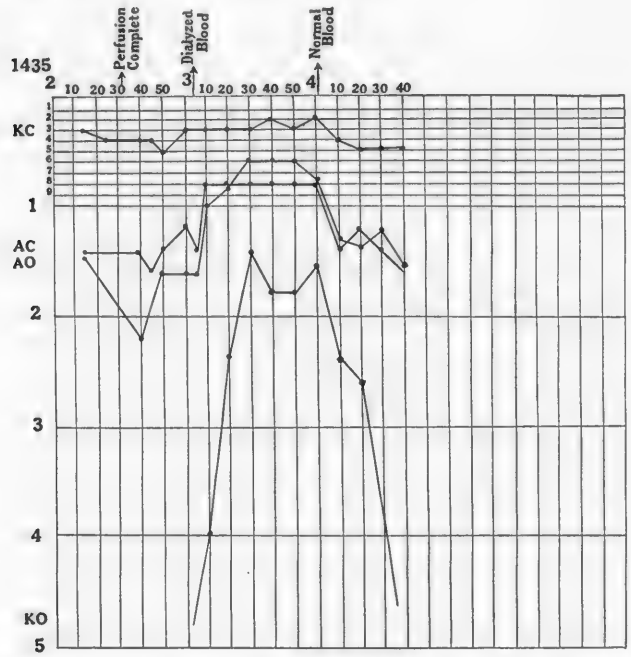
Sodium chloride	60.0
Sodium bicarbonate	5.0
Potassium chloride	4.0
Magnesium chloride	2.3
Dextrose	11.0
Calcium lactate	6.0
Water	10,000

	3.10		3.25	3.35	3.45	3.55	4.00	4.10	4.20	4.30	4.40	4.50	5.00	5.10	5.20
KC .	0.2		Perfusion	0.2	0.3	0.5	0.5	0.3	0.3	0.4	0.4	0.5	0.5	0.6	1.0
KO .	—			2.8	—	—	—	5.0	3.8	4.0	3.8	3.4	3.8	4.6	6.0
AC .	1.0			0.8	1.0	1.2	1.2	1.2	1.2	1.2	1.4	1.2	1.6	1.6	2.0
AO .	1.4			0.4	1.4	1.4	1.6	1.2	1.0	0.8	1.0	1.2	1.6	1.2	2.0

It will be seen from text-figure 2 that during the perfusion of normal blood a partial clamping off of the supply tube produced a

brief rise in excitability which then passed away when the current was turned on in its full strength.

Therefore, from that point the experiments were begun again with a dialyzing fluid containing neither calcium nor any considerable quantity of the disturbing phosphates. Probably with this fluid the blood lost some of its phosphates, but the difficulty of devising a dialyzing fluid which should remove nothing but calcium from the blood seemed insurmountable.[5]



TEXT-FIG. 3. Perfusion of leg with blood dialyzed against fluid devoid of calcium.

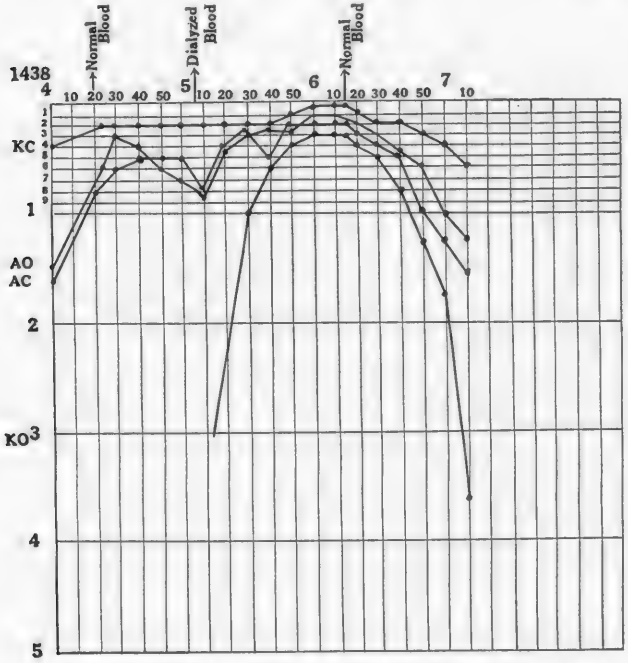
Dog 1,435.—(Text-figure 3.) Normal leg perfused at 2.32 P. M. with normal blood, at 3.01 with dialyzed blood, and at 4.01 again with normal blood. 900 c.c. of normal blood were dialyzed for 7½ hours against 18,000 c.c. of fluid (fluid 3) of the following composition, which was used in all later experiments.

[5] Since writing this paper it has been suggested to us by Dr. A. E. Taylor that as the phosphates in the blood are in non-dialyzable combinations there is little source of error in using a dialyzing fluid without phosphates.

Sodium chloride	60.0
Sodium bicarbonate	5.0
Magnesium chloride	2.30
Potassium chloride	4.0
Dextrose	10.9
Water	10,000

	2.15	2.18	2.32	2.40	2.45	2.50	3.00	3.05	3.10	3.20	3.30	3.40	3.50	4.00	4.10	4.20	4.30	4.40
KC	0.3	0.4	Perfusion complete	0.4	0.4	0.5	0.4	0.3	0.3	0.3	0.3	0.2	0.3	0.2	0.4	0.5	0.5	0.5
KO	—	—		—	—	—	—	—	4.0	2.4	1.4	1.8	1.8	1.6	2.4	2.6	—	4.0
AC	1.1	0.8		1.4	1.6	1.4	1.2	1.4	0.8	0.8	0.8	0.8	0.8	0.8	1.4	1.2	1.4	1.6
AO	1.4	1.0		2.2	2.0	1.6	1.6	1.6	1.0	0.8	0.6	0.6	0.6	0.8	1.4	1.4	1.2	1.6

Dialyzed blood — Normal blood



TEXT-FIG. 4. Perfusion of leg with blood dialyzed against fluid devoid of calcium.

Dog 1,438.—(Text-figure 4.) Normal leg perfused at 4.20 P. M. with normal blood. This was changed to dialyzed blood at 5.02, and again to normal blood at 6.12. The dialyzed blood was 1,000 c.c. which had been poured through the long celloidin tubules for 7 hours against 24,000 c.c. of the fluid described above.

	4.00	4.20	4.25	4.30	4.40	4.50	5.00	5.05	5.10	5.20	5.30	5.40	5.50	6.00	6.10	6.20	6.30	6.40	6.50	7.00	7.10
KC..	0.4	Perfusion complete	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.05	0.05	0.1	0.2	0.2	0.3	0.4	0.6
KO..	—		..	—	—	—	—	—	—	—	1.0	0.6	0.4	0.5	0.3	0.4	0.5	0.8	1.2	1.8	3.6
AC..	1.4		0.4	0.6	0.5	0.5	0.5	0.8	0.8	0.4	0.2	0.5	0.2	0.2	0.2	0.3	0.4	0.5	1.0	1.2	1.4
AO..	1.4		0.6	0.3	0.4	0.6	0.7	0.8	0.8	0.4	0.2	0.2	0.2	0.1	0.1	0.2	0.3	0.3	0.6	1.0	1.2
			↓ Normal blood					↓ Dialyzed blood								↓ Normal blood					

On the entrance of the dialyzed blood the leg jerked and moved itself about. Twitchings were evident until about 5.20.

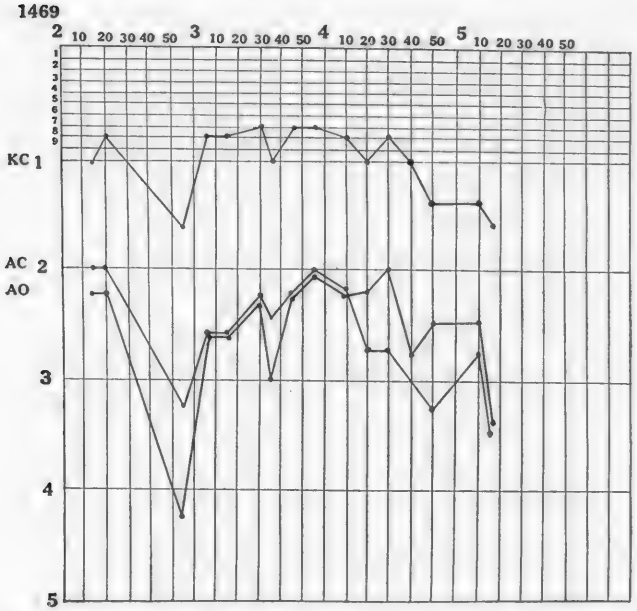
The chemical analysis of the dialyzing fluid before and after dialysis, and of the blood at various points in the experiments, is as follows. The figures show the amount of calcium per 1,000 c.c.

	Dog 1,435.	Dog 1,438.
Dialyzing fluid before dialysis of blood	0.0040	0.0043
Dialyzing fluid after dialysis of blood (1st)	0.0076	0.0076
Dialyzing fluid after dialysis of blood (2d)	0.0048	0.0054
Blood before dialysis	0.0364	0.0442
Blood after dialysis	0.0284	0.0233
Blood after perfusion of leg	0.0212(?)	0.0300

Text-figures 3 and 4 show clearly that perfusion with blood partly deprived of its calcium by dialysis produces great hyperexcitability of the nerves, and further that this must be due solely to the loss of calcium, since exactly the same procedure, changed only by the addition of precisely enough calcium to the dialyzing fluid to prevent any loss or gain of calcium by the blood, brings about no such hyperexcitability of the nerves. This proof may be somewhat strengthened by the addition of another curve made in the same way, except that the dialyzing fluid was so devised as to remove a large part of the chlorides from the blood, leaving the calcium unchanged.

Dog 1,469.—(Text-figure 5.) Normal leg perfused at 2.45 P. M. with normal blood, and at 3.30 with dialyzed blood. Dialysis was of 800 c.c. of normal blood for 6½ hours against 30,000 c.c. of fluid 4 of the following composition:

Sodium acetate	50.0
Sodium nitrate	40.0
Sodium bicarbonate	10.0
Magnesium chloride	2.3
Potassium nitrate	5.25
Calcium lactate	5.0
Dextrose	20.0
Water	10,000



TEXT-FIG. 5. Leg perfused with blood dialyzed against a chloride-free fluid.

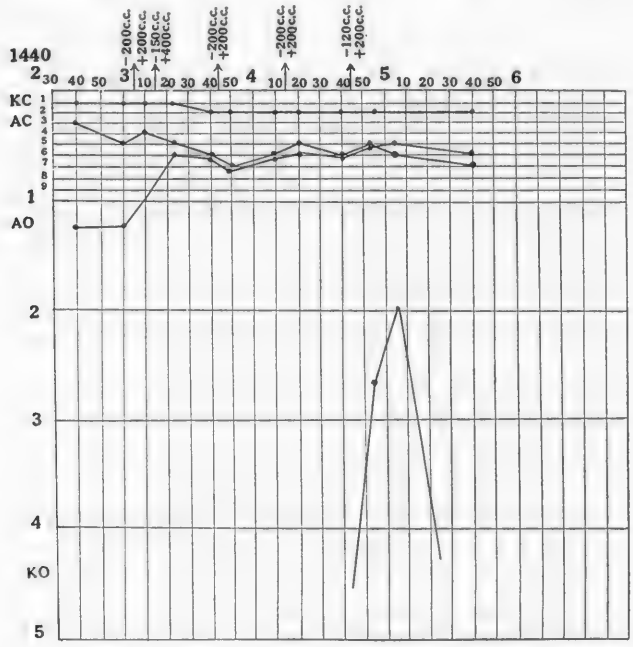
	2.15	2.20	2.45		2.55	3.05	3.15	3.30	3.35	3.43	3.55	4.10	4.20	4.30	4.40	4.50	5.10	5.15
KC...	1.0	0.8	Perfusion	Normal blood	1.6	0.8	0.8	0.7	1.0	0.7	0.7	0.8	1.0	0.8	1.0	1.4	1.4	1.6
KO...	—	—			—	—	—	—	—	—	—	—	—	—	—	—	—	—
AC...	2.0	2.0			3.2	2.6	2.6	2.2	2.4	2.2	2.0	2.2	2.8	2.8	3.0	3.2	2.8	3.4
AO...	2.2	2.2			4.2	2.6	2.6	2.2	3.0	2.2	2.0	2.2	2.2	2.0	2.8	2.6	2.6	3.4

Dialyzed blood

It is seen from text-figure 5, in which the variations are so slight, that hyperexcitability of the nerves is not produced by long perfusion, even with blood greatly altered by dialysis, so long as the dialysis has not removed the calcium. When the calcium is diminished, however, the effect in producing hyperexcitability is prompt and persists as in the case of tetany blood so long as blood altered in this way is passing, giving way to normal conditions as soon as normal blood is again circulated.

When an isolated extremity is perfused the influence of the undiluted dialyzed blood must be felt by the nerves, since there is but little tissue from which calcium or other constituents could be

absorbed in the passage of the blood. We thought, therefore, that this might be the most trustworthy expression of the nature of the influence of the dialyzed blood. Nevertheless we attempted to introduce dialyzed blood in quantity into the veins of an animal, realizing that it would at once meet with an abundant source of calcium in the tissues, but hoping to circulate it so rapidly as to surprise the nervous system and produce some hyperexcitability not only of the peripheral nerves but of the ganglion cells. We planned not only to wash out all the animal's blood with dialyzed blood but to replace that, too, with fresh dialyzed blood. Indeed after one or two experiments we even circulated fresh dialyzed blood through the whole animal, so that if the tissues were to supply calcium to all the new blood presented to them they must furnish enough to bring back to normal the calcium content of five or six times as much blood as was possessed by the animal before. At the beginning of each



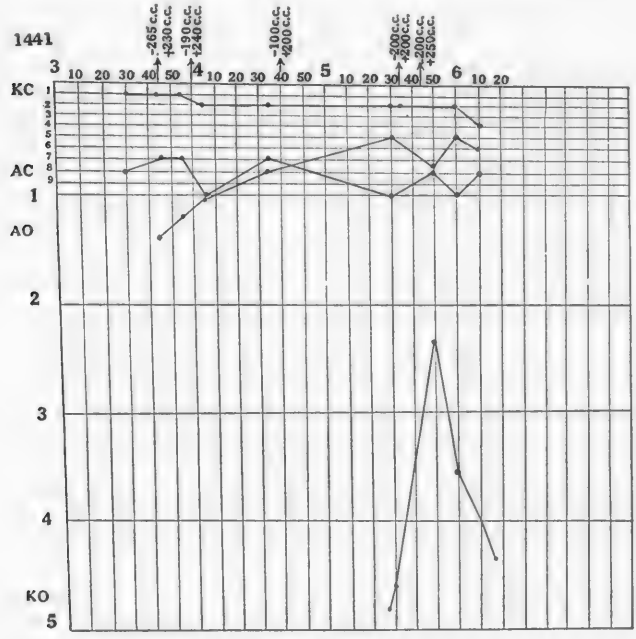
TEXT-FIG. 6. Repeated bleeding from carotid with replacement of blood dialyzed until poor in calcium.

experiment the parathyroids of the animal were removed. The protocols are as follows:

Dog 1,440.—(Text-figure 6.) Normal animal bled at intervals, dialyzed blood being returned. Thyroids and parathyroids removed at 4.35 P. M. No tetany developed.

	2.40	3.00	3.10	3.25	3.40	3.50	4.10	4.20	4.45	4.55	5.05	5.40
KC.	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
KO.	—	—	—	—	—	—	—	—	—	2.6	2.0	—
AC.	0.3	0.5	0.4	0.5	0.6	0.7	0.6	0.5	0.6	0.5	0.6	0.7
AO.	1.2	1.2	1.0	0.6	0.6	0.7	0.6	0.6	0.6	0.5	0.5	0.6
Bled		200 c.c.	150 c.c.				200 c.c.		120 c.c.			
Returned dialyzed blood		200 c.c.	400 c.c.				200 c.c.		200 c.c.			

In this experiment 1,350 c.c. of blood dialyzed for 7 hours against 30,000 c.c. of fluid 3 were used in replacing normal blood. Effect very slight.



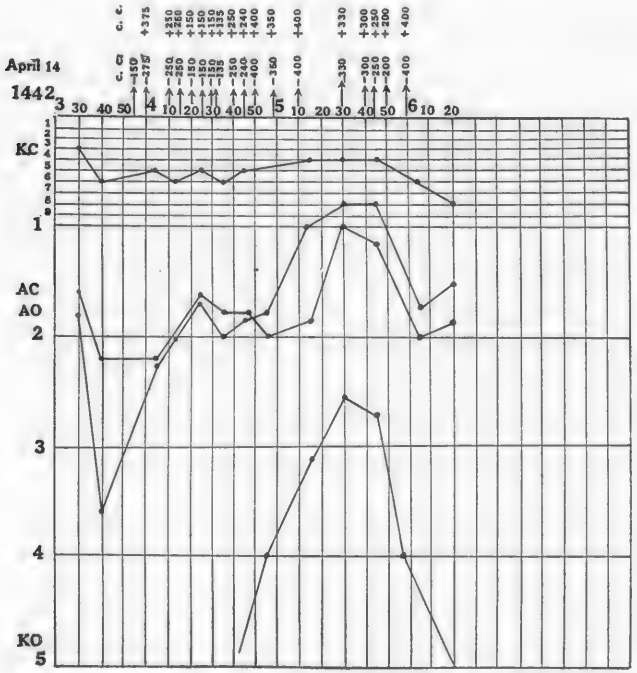
TEXT-FIG. 7. Normal animal. Blood replaced at intervals with dialyzed blood.

Dog 1,441.—(Text-figure 7.) Normal animal, blood at intervals replaced with dialyzed blood. 1,300 c.c. of blood dialyzed for 7½ hours against 26,000 c.c. of fluid 3. Parathyroids were removed just before the experiment started.

	3.30	3.45	3.55	4.05	4.35	5.30	5.50	6.00	6.10
KC .	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.4
KO .	—	—	—	—	—	4.6	2.4	3.6	4.0
AC .	0.8	0.7	0.7	1.0	0.7	1.0	0.8	1.0	0.8
AO .	1.4	1.4	1.2	1.0	0.8	0.5	0.8	0.5	0.6

Bled	265 c.c.	190 c.c.		200 c.c.	200 c.c.
Returned dialyzed blood	230 c.c.	240 c.c.		200 c.c.	250 c.c.

There is general twitching; no definite tetany; much vomiting.



TEXT-FIG. 8. Normal animal through which dialyzed blood was actually circulated.

Dog 1,442.—(Text-figure 8.) Normal animal through whose blood vessels dialyzed blood was actually circulated, as shown in the chart. Parathyroids first removed.

	3.20	3.40	4.05	4.12	4.25	4.35	4.45	4.55	5.15	5.30	5.45	6.05	6.20
KC...	0.3	0.6	0.5	0.6	0.5	0.6	0.5	0.5	0.4	0.4	0.4	0.6	0.8
KO...	—	—	—	—	—	—	—	4.0	3.2	2.6	2.8	4.0	5.0
AC...	1.6	2.2	2.2	2.0	1.6	1.8	1.8	2.0	1.8	1.0	1.2	2.0	1.8
AO...	1.8	3.6	2.2	2.0	1.6	2.0	1.8	1.8	1.0	0.8	0.8	1.8	1.6

Bled	150 c.c.	275 c.c.	250 c.c.	250 c.c.	150 c.c.	150 c.c.	135 c.c.	250 c c.	240 c.c.	400 c.c.	350 c.c.	400 c.c.	330 c.c.	300 c.c.	250 c.c.	200 c.c.	400 c.c.		
Returned dialyzed blood	375 c.c.		250 c.c.	250 c.c.	150 c.c.	150 c.c.	135 c.c.	250 c.c.	240 c.c.	400 c.c.	350 c.c.	400 c.c.	330 c c.	200 c.c.	250 c.c.	200 c.c.	400 c.c.		

1,800 c.c. of blood dialyzed for 7 hours against 30,000 c.c. of fluid 3 were used. Each part of this probably circulated two and one half times. Vomiting; no definite twitching; collapse and death.

From these experiments it seems clear that it is impossible in a short time to reduce the calcium content of the blood circulating through the whole body or to keep it reduced to such a degree as to bring about a tetany-like hyperexcitability of the nerves. There is too abundant a source for fresh calcium in the various tissues, including the bones, and the blood quickly replenishes itself. It might seem possible to repeat this process during several days and thus imitate the latent period which follows extirpation of the parathyroids, but in this case it would be necessary to extirpate those glands, when tetany would arise of itself, since otherwise the blood would rapidly replenish its calcium, under their influence, from the skeleton. Indeed, the rapidity with which calcium enters the circulating dialyzed blood from the tissues is shown by comparison of the analyses of samples taken before and after its circulation in the body.

In dog 1,441 the blood contained per 1,000 c.c.:

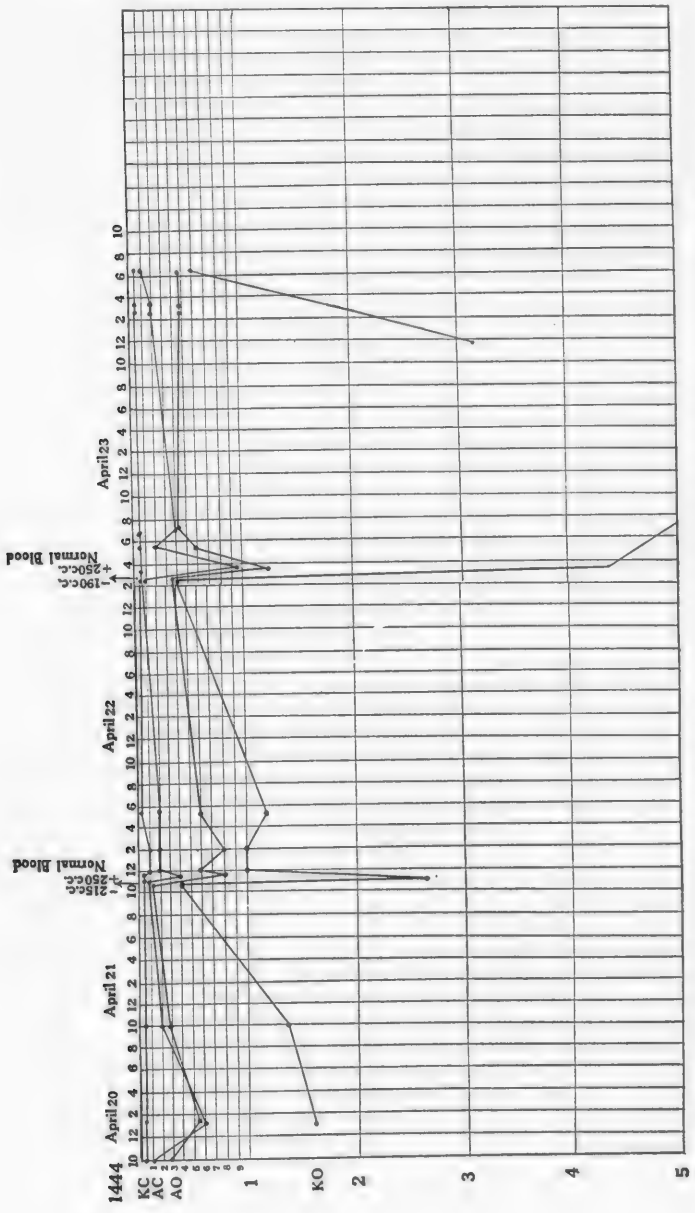
Before dialysis	0.0485
After dialysis	0.0190
After circulation for about 2 hours	0.0381

It remains a question whether, if one could continue this depletion over several days, it would be possible to produce such changes in excitability as are seen in tetany, and in fact this was our original problem. It leaves one with the impression that the parathyroid secretion must govern the conversion of a non-dialyzable and useless combination of calcium into a dialyzable form which is essential for the control of the excitability of the nerves, but which is lost

in the excreta and not newly formed in the absence of parathyroid secretion.

Although we had learned that it is possible by perfusion with calcium-poor blood to produce hyperexcitability of the nerve, it remained to bring this fact into direct relation with the condition in tetany, for it might be objected that there the mechanism is a different one. This we thought would be attained if we could show that this dialyzed blood had lost the power possessed by normal blood of curing tetany on transfusion. It is well known that if an animal in tetany, after the removal of the parathyroids, be bled and the blood replaced with normal blood, the excitability of the nerves falls, and twitchings disappear for a time. Dialyzed blood poor in calcium has little or nothing of this effect, although if the dialyzed blood be not so poor in calcium as that of the animal in tetany, it will lower the excitability somewhat. If in an animal in tetany we replace the blood with dialyzed blood and find no change in the symptoms nor lowering of the excitability, we may remove that blood and replace it with normal blood which does stop the twitching and lower the excitability.

But if, although the excitability is not lowered by the dose of dialyzed blood, we do nothing further, the excitability is found to be lowered the next day or after some hours. To explain this we have imagined that while in dialysis we remove the calcium from the normal blood, we may not remove the parathyroid secretion which on being injected into a dog in tetany after a time causes the appearance of dialyzable calcium in the blood and goes far to relieve temporarily the tetany. It is on this basis that it seems hopeless to attempt to produce a condition resembling tetany by the repeated replacement of the blood over a period of days with normal blood dialyzed until it is poor in calcium, because at the same time we furnish parathyroid secretion which ensures the continual withdrawal of calcium from the tissues. It is true that we might dialyze tetany blood, but even then having a blood free from parathyroid secretion and poor in calcium we have no animal in which to make evident the effect of pure lack of calcium, for the animal with intact parathyroids will continually frustrate this object, and the animal deprived of parathyroids will develop tetany during the experiment.

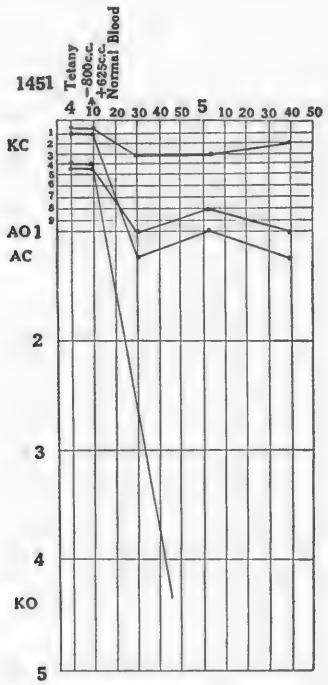


TEXT-FIG. 9. Tetany. Bled on two occasions; normal blood replaced. Transient effect.

ILLUSTRATIVE PROTOCOLS.

Dog 1,444.—(Text-figure 9.) Tetany developing 2 days after parathyroidectomy. Bled on two occasions, normal blood being replaced. Transient relaxation and lowering of excitability.

	April 20.		April 21.						April 22.				April 23.		
	10.00	1.45	10.00	11.15	11.40	12.00	2.00	5.45	2.30	2.55	5.30	6.35	2.20	3.10	6.30
KC	0.05	0.05	0.05	0.05	0.05	0.1	0.1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
KO	.....	1.6	1.4	0.4	2.6	1.0	1.0	1.2	0.4	4.4	—	—	—	—	0.6
AC	0.1	0.6	0.2	0.05	0.4	0.2	0.2	0.2	0.05	1.0	0.2	0.4	0.2	0 2	0.1
AO	0.3	0.6	0.3	0.1	0.8	0.6	0.8	0.6	0.4	1.2	0.6	0.4	0.5	0.5	0.5
Bled					215 c.c.					190 c.c.					
Returned normal blood					250 c.c.					250 c.c.					



TEXT-FIG. 10. Tetany. Effect of bleeding and replacing normal blood.

Dog 1,451.—(Text-figure 10.) Violent tetany 24 hours after parathyroidectomy. Bled 800 c.c., and this loss was replaced by 625 c.c. of normal blood. Immediate relaxation and lowering of excitability of nerves.[6]

[6] A curious phenomenon was observed in these cases in which nearly all the

	April 26.					April 27.	April 28.
	4.00	5.00	5.20	5.55	6.30	11.00	12.15
KC.....	0.05	Blood replaced by normal	0.3	0.3	0.2	0.1	0.05
KO.....	0.4		—	—	—	—	0.3
AC.....	0.1		1.2	1.0	1.2	1.2	0.05
AO.....	0.4		1.0	0.8	1.0	1.2	0.2

Bled 800 c.c. 425 c.c.
Returned normal blood 625 c.c. Returned dialyzed blood 400 c.c.

This animal developed tetany again on April 28 and was bled 425 c.c., 400 c.c. of dialyzed blood being replaced. There was no recovery from the spasm brought on by the bleeding, and death followed rapidly. This case forms an example of the contrast between the effects of introducing normal and dialyzed blood.

Dog 1,445.—Moderate tetany 2 days after parathyroidectomy. At 2.23 P. M. bled 150 c.c. Blood replaced by 150 c.c. of blood dialyzed for 5 hours against 10,000 c.c. of fluid 3. At 3.20 bled 150 c.c., and replaced 150 c.c. of normal blood. At 10.30 bled 200 c.c., and replaced 250 c.c. of normal blood.

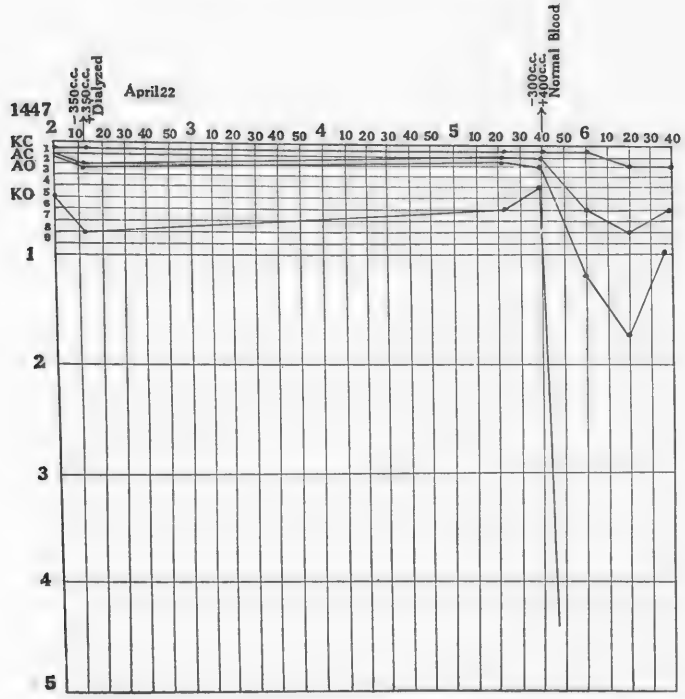
	April 20.									April 21.						
	1.30	1.45	2.20	2.30	2.40	3.00	3.20	3.45	4.25	10.00	10.45	11.00	11.15	12.00	2.00	5.45
KC.	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
KO.	0.2	0.3	0.3	0.3	0.4	0.3	0.6	1.4	0.6	0.2	0.4	0.4	0.4	—[7]	2.0	1.4
AC.	0.05	0.1	0.05	0.05	0.05	0.05	0.1	0.2	0.1	0.05	0.05	0.1	0.1	0.1	0.2	0.3
AO.	0.2	0.3	0.3	0.3	0.4	0.3	0.4	0.5	0.3	0.3	0.4	0.5	0.4	0.4	0.3	0.3

Bled 150 c.c. 150 c.c. 200 c.c.
Returned dialyzed blood 150 c.c. Returned normal blood 150 c.c. 200 c.c.

Dog 1,447.—(Text-figure 11). Violent tetany at 2 P. M. Bled 350 c.c. which were replaced with 350 c.c. of blood from a lot of 600 c.c. which had been dialyzed for 4½ hours against 12,000 c.c. of fluid 3. Change in character of electric reaction, but no marked change in excitability. Contraction slow, no rigidity. Tongue tremor and twitchings continue. At 5.45 bled 300 c.c., and replaced 400 c.c. of normal blood. Complete relaxation and return of excitability to normal.

blood was removed, at the moment before the normal blood was allowed to run in. Although twitching may have been slight when the bleeding began, the most violent contractions and extreme rigidity appeared during the extreme anemia, to disappear at once when the other blood entered the veins. Probably this is due to temporary asphyxia of the nerves.

[7] Negative up to 3 milliamperes.



TEXT-FIG. 11. Tetany. Bleeding on two occasions. Dialyzed blood replaced without effect. Normal blood reduces excitability.

	April 22.										
	11.45	2.00	2.15	2.55	5.25	5.40	6.00	6.20	6.45	9.00	12.00
KC..	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.2	0.2	Quiet;	Twitch-
KO..	0.5	0.5	0.8	Tongue and muscle	0.6	0.4	—	—	—	no tongue	ing; tremor in
AC..	0.1	0.1	0.2	tremor	0.1	0.1	0.6	0.8	0.6	tremor	tongue.
AO..	0.1	0.1	0.2		0.1	0.2	1.2	1.8	1.0		
Bled			350 c.c.				300 c.c.				
Returned dialyzed blood			350 c.c.			Returned normal blood			400 c.c.		

Dog 1,452.—No tetany 4 days after parathyroidectomy. Moderately high excitability. Bled 315 c.c., replaced by 330 c.c. of blood dialyzed 7 hours against fluid 3, $\frac{430 \text{ c.c. blood}}{10{,}000 \text{ c.c. fluid}}$. Animal much depressed and vomited. Excitability a

little lowered, and this lowering continued and increased for the next two days. Then bled 300 c.c., which were replaced by 300 c.c. of blood dialyzed for 6 hours, $\frac{400 \text{ c.c.}}{15{,}000 \text{ c.c.}}$. No marked change in excitability.

	April 29.					May 1.				
	5.00	5.25	5.35	5.50	6.05	2.00	5.00	5.40	5.50	6.25
KC.....	0.05	0.2	0.2	0.2	0.1	0.2	0.1	0.2	0.2	0.1
KO.....	1.8	2.6	2.0	2.0	2.0	—	—	—	—	—
AC.....	0.4	0.8	0.8	1.2	1.0	1.4	1.2	1.2	1.2	1.2
AO.....	0.5	0.8	0.8	1.0	0.8	2.0	1.4	1.4	1.6	2.0
Bled		315 c.c.					300 c.c.			
Returned dialyzed blood		330 c.c.					300 c.c.			

We made a number of analyses of the blood of animals in tetany, of the dialyzed normal blood used to replace their own, of the normal blood, and of the normal or dialyzed blood removed again after it had circulated for a time in the animal, thinking that a comparison of these figures might throw some light on what occurred in the body to influence the tetany. But such figures would be difficult to interpret because they would change continually with the time the new blood was allowed to stay in the body, first through the process of obtaining a balance with the tissues, and then through the same process that disturbed the composition of the blood to produce tetany. It might be expected then that thoroughly dialyzed blood left in a tetany animal would become richer in calcium for awhile. although normal blood introduced in the same way would steadily lose calcium until it became very poor indeed, when another attack of tetany might come on, as in dog 1,451.

If tetany blood be dialyzed under exactly the same conditions as normal blood, it still loses a proportionate amount of its calcium, which would perhaps show that it is not especially the loss of a diffusible calcium as contrasted with a non-diffusible form which is important in producing tetany. The blood of dog 1,451 taken during tetany contained 0.0287 of a gram of calcium per 1,000 cubic centimeters. 500 cubic centimeters dialyzed for 5 hours against 12,000 cubic centimeters of fluid 3 then contained 0.0114 of a gram per 1,000 cubic centimeters.

CONCLUSIONS.

We may devise a fluid containing practically all the inorganic diffusible constituents of the blood except calcium, and use it to dialyze normal blood in such a way as to remove from it a large part of its calcium. The dialyzed blood when perfused through an isolated extremity produces an extreme hyperexcitability of the nerves quite like that observed in tetany. Since perfusion with blood dialyzed in precisely the same way against a fluid of the same composition, but containing calcium in the proportion found in the normal blood, causes no hyperexcitability of the nerves, it is evident that the hyperexcitability is due to the lack of calcium. This effect can be attained in only a slight degree by replacing the blood of a whole animal with the dialyzed blood, since under the conditions of the experiment the tissues cannot be sufficiently depleted of their calcium. It seems probable that the parathyroid secretion is not removed by dialysis, but is returned to the body with the dialyzed blood.

To bring this result into relation with the condition in tetany following parathyroidectomy, animals in tetany were bled and the blood was replaced in one case with normal blood, in the other with dialyzed blood poor in calcium. The normal blood immediately relieves the tetany and lowers the excitability, while the dialyzed blood does not. We therefore believe that this is a further proof that in the tetany of parathyroidectomy also the twitching and hyperexcitability of the nerves is due to lack of calcium in the blood and tissues.

THE EXPERIMENTAL PRODUCTION OF NECROSIS OF THE LIVER IN THE GUINEA PIG.*

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The starting point for our experiments was the observation that intravenous injections of various substances, as leech extract, colloidal solutions of metals, casein, and nucleoproteid caused an inhibition in the growth of mouse tumors.[1] In order to determine the mechanism through which these substances act, we studied their actions on the organs of guinea pigs, and we noticed that a single intravenous injection of solutions of various proteids and of hirudin apparently caused necrosis in the liver of the injected animals. In the mouse, on the other hand, we did not observe this effect on the liver. Observations concerning the experimental conditions under which necrosis of the liver is produced in the guinea pig are complicated through the apparently spontaneous occurrence of necrosis in the liver of these animals.

Mallory,[2] studying necrosis in the human liver, reports on some experiments in the guinea pig in which he found that various experimental procedures, as massage of the spleen, pressure exerted on the liver, subcutaneous injection of diphtheria toxin, or the passing of a galvanic current through the abdomen of an animal, apparently caused necrosis in the liver. He appears doubtful, however, as to the interpretation of these observations. He noticed this necrosis two hours as well as twenty-four hours after the experimental interference, and furthermore found necrosis in untreated control animals. He comes, therefore, to the conclusion that the guinea pig is entirely unsuited for the study of necrosis of the liver. Inasmuch as no data were given in Mallory's work which make possible a comparison between the number and extent of necroses of the

* Received for publication, May 18, 1914.

[1] Loeb, L., and Fleisher, M. S., *Jour. Am. Med. Assn.*, 1913, lx, 1857.

[2] Mallory, F. B., *Jour. Med. Research*, 1901, vi, 264.

TABLE I.

	Injected with 10 c.c. of 1⅔% solution of casein.	Injected with 2½ c.c. of 15% solution of serum globulin.	Injected with 2 c.c. of 15% solution of nucleoproteid.	Injected with 4–8 c.c. of ¼% solution of hirudin.	Injected with 10 c.c. of colloidal copper.	Injected with 10 c.c. of distilled water.	Injected with 5 c.c. of 15% solution of ovalbumin.	Injected with 3 c.c. of 5% solution of protamin.	Injected with 10 c.c. of 5% solution of starch.	Injected with strophanthin 0.039 mgm. in 10 c.c. of water.	Controls uninjected.
No. of animals....	87	27	51	43	53	24	16	20	21	20	297
No lesion.....	15 (17%)	4 (15%)	8 (16%)	5 (11%)	9 (17%)	3 (12%)	6 (38%)	6 (30%)	9 (43%)	4 (20%)	192 (64%)
1 very small lesion.	8 (9%)	6 (22%)	3 (6%)	2 (4%)	4 (7%)	1 (4%)	1 (6%)	2 (10%)	2 (10%)	2 (10%)	30 (10%)
2 or 3 very small lesions.....	8 (9%)	1 (4%)	9 (18%)	4 (9%)	3 (6%)	7 (29%)	2 (12%)	.....	4 (19%)	1 (5%)	22 (7%)
Few small or larger lesions.....	18 (20%)	3 (11%)	4 (8%)	10 (23%)	11 (21%)	4 (17%)	3 (19%)	5 (25%)	3 (14%)	5 (25%)	27 (9%)
Multiple lesions...	38 (47%)	13 (48%)	27 (52%)	24 (53%)	26 (49%)	9 (38%)	4 (25%)	7 (35%)	3 (14%)	8 (40%)	26 (9%)
Typical lesions (included among multiple lesions).	14 (1 %	7 (26%)	14 (28%)	9 (21%)	10 (19%)	2 (8%)	.....	2 (10%)	1 (5%)	1 (5%)	4 (1⅓%)

liver in control animals and in animals subjected to various experimental procedures, this conclusion of Mallory's appears to be justified.

In order to decide definitely how far the necrosis which we found in the livers of guinea pigs was caused by our experimental procedures, and how far it was apparently due to other spontaneous factors not analyzed, it was necessary to determine first the frequency and extent of necroses of the liver in a large number of control animals.

For this purpose we examined the livers of 297 guinea pigs, some of which served also as controls for the animals used for intravenous injections of various substances. They were usually animals obtained from the same breeder and at the same time as the animals which were injected. We added as controls a number of animals which had been used in other experiments, especially animals in which incisions had previously been made in the uterus and ovaries.

The last columns of tables I and II show the frequency and extent of the necroses in the liver of these control animals.

TABLE II.

	Group A.	Group B.	Controls.
No. of animals	261	101	297
No lesion	41 (16%)	28 (28%)	192 (64%)
1 very small lesion	23 (8%)	8 (8%)	30 (10%)
2 or 3 very small lesions	25 (10%)	14 (13%)	22 (7%)
Few small or large lesions	46 (17%)	20 (20%)	27 (9%)
Multiple lesions	128 (49%)	31 (31%)	26 (9%)
Typical lesions (included among multiple lesions)	54 (21%)	6 (6%)	4 (1⅓%)

We see that 64 per cent. of the control guinea pigs were free from lesions, while 17 per cent. of the remaining animals had only very slight necrosis. 9 per cent. had a few small or larger necrotic areas, and only 9 per cent. had marked multiple lesions. Of these latter animals 1⅓ per cent. had the typical multiple necrosis in which certain more or less extensive parts of the livers were dotted with small necrotic spots between which at some places larger conglomerate necrotic areas could be found.

If we analyze further the occurrence of necrosis in the liver of

the control animals, all but one of the cases in which multiple lesions were found occurred in animals which had been examined either within five days following an operation (usually affecting the ovaries or uterus), or one or two days after arrival from a railway journey lasting more than twenty-four hours, and during the hot summer months. In these two classes of control animals exclusively the severe (typical) lesions were found. Many control animals were examined twelve to fifteen days after an operation; these were in most cases free from macroscopical lesions of the liver (table III).

TABLE III.

	Total.	Examination within 5 dys. of operation.	Sick preceding death.	Day after arrival on which examined.	Bled.
No lesion	4	2	1	1	–
1 very small lesion	6	5	–	–	1
2 or 3 very small lesions	4	1	1	2	–
Few small or larger lesions	8	3	1	4	–
Multiple lesions	17	7	1	9	–
Typical lesions (included among multiple lesions)	4	1	–	3	–

If we compare with these control animals, guinea pigs which received, usually twenty to twenty-eight hours before examination, an intravenous injection of a solution of casein, nucleoproteid, serum globulin, hirudin, or of colloidal copper, we find very much more marked necrosis. The difference between these groups and the control animals becomes especially clear if we compare the frequency of severe lesions in both classes of animals. In table II under the heading of group A, the average frequency of 261 animals is given. Marked multiple lesions were observed in almost one half of the animals, and in not quite one half of the animals showing multiple lesions the liver showed over certain areas the typical dotted appearance. On the other hand, in animals injected with distilled water, solutions of egg albumin, protamin, starch, and strophanthin (group B), the lesions, although much more marked than in control animals, were distinctly less than in those animals united under group A. In group B especially the severe lesions are less marked than in group A. Altogether we examined 659 guinea pigs. Considering the large number of the animals examined our

results are sufficiently concordant to present the following conclusions: (1) In a certain percentage of guinea pigs there occurs apparently spontaneous necrosis of the liver. This necrosis is very much more severe and frequent in animals subjected to injurious influences, as to a long journey in hot weather, or to an abdominal operation. Within a few weeks after the exposure to the injurious influence, the majority of the necroses seem to disappear. In healthy animals not subject to these injurious influences, necrosis of the liver is on the whole not frequent, although small lesions may occasionally occur. (2) Intravenous injections of a great variety of substances dissolved in water, and even of distilled water cause a marked increase in the necrotic lesions found in guinea pigs. It seems, however, that different substances show an unequal tendency to produce these lesions.

Microscopical Examination of the Lesions.—Most lesions were examined approximately one day after the injection of the various substances. Some, however, were examined at an earlier period; others, from six to ten days after the injection. Many lesions in control animals were also examined. We found the lesions in the beginning situated somewhere between the portal and central areas. If the lesions were larger they sometimes extended to the portal or central veins. At first the lesions were isolated and well defined. If they became more extensive neighboring lesions could join and form conglomerate lesions. Small lesions consisted of a single cell or of a few cells with somewhat shrunken, dense nuclei and hyaline cytoplasm which stained markedly with eosin. In larger lesions and at a later period, at first the center and later the greater part of the necrotic area showed signs of autolysis, the nuclei disappeared, the cytoplasm became vacuolar, and in the end a network of fibers remained. Very soon polynuclear leucocytes collected in the necrotic areas. At first they were situated in the capillaries, later they penetrated into the cells and helped to dissolve them. There were also visible occasionally some cells which probably represented swollen endothelial cells of the capillaries of the liver or a few isolated immigrated connective tissue cells. Thrombi occluding the vessels were absent, if we do not regard collections of polynuclear leucocytes in the capillaries, the number of which, however, varied

exceedingly at different places and in different specimens, as representing cell thrombi. It is probable that the collection of the polynuclear leucocytes is a secondary phenomenon, the necrosis of some liver cells being the primary change which caused the leucocytes to collect around the diseased cells. Connective tissue cells begin to grow into the necrotic areas, and in pieces taken out six days after the injection the nuclei in the necrotic areas have faded; connective tissue has supplanted a considerable part of the material. The ingrowing connective tissue cells may assume the shape of epithelioid cells. Proliferating bile ducts may be visible in the growing connective tissue. Ten days after the injection areas of newly formed connective tissue with dilated blood vessels and proliferated bile ducts and with some polynuclear leucocytes are visible. The necrotic material has entirely disappeared at that period. Hemorrhages are frequently visible in and around the necrotic areas; even ten days after the injection there may still be present some signs of hemorrhage. They are perhaps especially marked after injection of hirudin. At other times, however, hemorrhages may be absent.

We may conclude from the microscopical studies (1) that thrombi are not responsible for the origin of the necrosis, (2) that the lesions have usually an intermediate position, and (3) that they become organized through connective tissue.

Notwithstanding their substitution by connective tissue and the relative frequency of these lesions, they evidently do not lead to cirrhosis of the liver. Cirrhotic changes in the liver of the guinea pig must be extremely rare. It seems that after a few weeks these lesions disappear entirely or almost entirely, without causing retractions at the surface of the liver, notwithstanding the fact that they are situated preferably near the surface of the liver. In our investigations into experimental myocarditis we also had indications of the apparent gradual disappearance of the lesions. For the production of cirrhosis there must perhaps occur extensive and repeated necrotic changes in a certain area.

The fact that so many different conditions have the same effect, causing focal necrosis of the liver, suggested the possibility that one common factor may be present in all of them. The idea suggested itself that the presence of bacteria in the circulation and their

subsequent retention in the liver might be responsible for the lesions. We searched, therefore, a certain number of animals showing necrosis of the liver for the presence of bacteria either in the blood or in the liver. We examined three control guinea pigs which showed necrotic spots in the liver. A platinum wire was pushed into the necrotic areas, withdrawn, and the adhering material distributed into bouillon tubes. In one case blood was withdrawn from the heart and mixed with sterile bouillon. The results were negative. In four animals in which through intravenous injection of colloidal copper necrosis of the liver had been produced, a similar bacteriological examination of the necrotic areas also proved negative. The colloidal solution of copper used for injection was found to be sterile. The results obtained after injection of a solution of hirudin were, however, positive. In five animals we examined the heart blood bacteriologically approximately one day after the injection of the hirudin, and we found bacteria in one animal. In nine animals we examined the necrotic spots in the liver for bacteria by means of cultural methods, and found them present in four animals. An examination of the hirudin solution used for injection showed the presence of apparently similar organisms to those found in the blood or necrotic spots of the liver. It is probable that in the case of hirudin bacteria were introduced into the circulation of the animals with the solution and that the bacteria afterwards lodged in the necrotic areas. We may, however, on the basis of our negative results in control animals and in animals injected with colloidal copper, conclude that in general bacteria are not responsible for the liver necrosis produced through the various experimental interferences.

An analysis of the shape and distribution of the necrotic area as it reveals itself to macroscopic examination aids us somewhat in the etiologic interpretation of these lesions. The edges and the superficial parts of the lobes of the liver are favored,—an observation made also by previous investigators in the case of other necroses of the liver. At these places the circulation is most easily interfered with, and interference with the circulation may, therefore, be suggested as one of the factors of importance in the causation of this necrosis. Frequently the necrosis has the shape of straight lines, sometimes

running almost parallel to the suspensory ligament of the liver. Occasionally they seem to be located at places where the ribs exert pressure on the liver. It is not uncommon for a lesion to be continued from one lobe to an adjoining one, as if the two lobes formed one connected whole. This is in some cases probably due to the fact that pressure is simultaneously exerted on two overlapping or adjoining lobes; in other cases, where bacteria cause the lesions, it may be due to a transmission of the organisms from one lobe to the neighboring one through contact.

The readiness with which this necrosis is produced seems to vary greatly in different species of animals. While in the guinea pig it is evidently easy to produce necrosis of the liver, it is much more difficult to do so in the mouse. In the mouse we did not observe necrosis produced by one injection of either colloidal copper or hirudin, as in the guinea pig. In one mouse we found necrotic spots in the liver after repeated injections of hirudin.

At present it is not possible to give a definite analysis of the factors causing necrosis in the liver of guinea pigs. Some are in all probability caused by certain bacteria lodging in the liver. But this explanation cannot apply to the majority of the necroses produced in our experiments. The various methods which we use have in all probability one or two factors in common, and in every case these cause the necrosis. We have indications that mechanical factors that weaken the circulation at certain places in the liver are at least partly responsible for the origin of the necrosis. It may be that injection of the various substances contributes to the production of the necrosis by reducing still further the pressure with which the circulation is maintained in the liver, or that these substances reduce the resistance of the liver cells to unfavorable conditions of the circulation by altering the metabolism of the liver cells. We are tempted to analogize the necrosis of the liver produced experimentally through these injections with the ulcer of the stomach produced by Rehfuss[3] in the guinea pig through the injection of venom of Heloderma and of various other substances. All that these substances have in common is a marked influence on the general circulation and vitality of the animal. It is probable that

[3] Rehfuss, M. E., *Carnegie Institution of Washington Publications*, 1913. No. 177, 125.

as a result of the weakening of the circulation the epithelial cells of the gastric mucosa become injured, lose their power of resistance to the action of the gastric juice, and are, therefore, digested by the latter. In both cases thrombi are not the primary cause of the lesions; hemorrhages are common to both lesions; in both cases very diverse substances can produce the same result and the common factor underlying the action of all of them is probably a reduction of the vitality of the cells either through lowering the blood pressure or through toxic interference with the metabolism of the cells. In both cases secondary factors are added,—mechanical factors (pressure) in one case, digestive action of the gastric juice in the other. We may add that the intravenous injection of hirudin causes in a certain number of cases not only necrosis of the liver but also gastric ulcers. Hemorrhage is probably the cause of these ulcers.

Necrosis of the liver has been produced experimentally in the liver of animals (Flexner,[4] Theobald Smith,[5] Boxmeyer,[6] Pearce,[7] Joannovics,[8] and Opie[9]). The necrosis observed by Flexner after injection of ricin showed much similarity to the necrosis observed by us in the liver of the guinea pig. Flexner found no definite relation between the necrosis and thrombi in the blood vessels supplying the necrotic area. He therefore doubts an etiological significance of thrombi which may occasionally be found in the affected areas; he thinks it possible, however, that lesions in the capillaries lead to an increased transudation of toxin-containing lymph and may thus be responsible for the focal necrosis in the liver. Pearce laid special stress on the occurrence of agglutinative thrombi in the vessels of the liver accompanying necrosis produced through intravenous injection of hemolytic serum, and he referred the necrosis in the main to interference with the circulation caused by these thrombi, although he admitted the possibility of other factors playing a part

[4] Flexner, S., *Johns Hopkins Hosp. Rep.*, 1897, vi, 359; *Jour. Med. Research*, 1902, viii, 316; *Med. News*, 1894, lxv, 116.

[5] Smith, T., and Moore, V. A., *U. S. Department of Agriculture, Bureau of Animal Industry*, 1894, *Bulletin No.* 6.

[6] Boxmeyer, C. H., *Jour. Med. Research*, 1903, ix, 146.

[7] Pearce, R. M., *Jour. Exper. Med.*, 1906, viii, 64.

[8] Joannovics, G., *Ztschr. f. Heilk.*, 1904, xxv, 25.

[9] Opie, E. L., *Jour. Exper. Med.*, 1910, xii, 367.

in their origin. One of us has shown[10] that the necrosis produced through intravenous injection of ether is due to two factors: (1) a direct injurious action of the ether on liver tissue, and (2) autolytic changes induced through clotting in the larger blood vessels. The presence of the first factor could be demonstrated in an exact manner through observation of the development of the necrosis *in vitro* under conditions where interference with the circulation no longer could determine the origin of localized, focal necrosis at the same places where the necrotic areas would have arisen in the living body. At a later date the extension of the necrosis took place in the area supplied by blood vessels thrombosed as a result of the primary action of the ether and of the primary necrosis. The clotted blood vessels at this period are no longer open to the passage of carmin particles injected into the vessels of the liver. Karsner and Aub[11] were not able to produce, through injection of hemolytic serum, the same effects as those obtained by us through injection of ether. This was not to be expected inasmuch as hemolytic serum acts very much less strongly than does ether introduced into the circulation. Karsner and Aub were, therefore, unable to determine the manner in which the injection of hemolytic serum produces necrosis in the liver. It may be suggested that possibly some light may be thrown on the relative significance of direct toxic action on the blood vessels and liver tissue and of secondary effects due to thromboses by an exact determination of the time relations in the development of the necrosis. It is to be expected that effects due to a direct toxic action appear earlier than the effects due to thrombosis, the latter proceeding approximately at the same rate as the autolytic processes due to complete ligation of a lobe of the liver.

SUMMARY.

Through the intravenous injection of various substances differing very much in character, multiple necrosis can be produced in the liver of the guinea pig. In the mouse the effect of these substances is absent or much less marked. Different substances seem to differ,

[10] Loeb, L., and Meyers, M. K., *Virchows Arch. f. path. Anat.*, 1910, cci, 78.
[11] Karsner, H. T., and Aub, J. C., *Jour. Med. Research*, 1913, xxviii, 377.

however, in their power to produce necrosis. In control animals necrosis in the liver is much more rare. It is found especially in animals subjected to various injurious influences. The necrotic areas are usually situated between the portal and central areas of the liver acini. Their development is not due to thromboses interfering with the circulation in certain areas of the liver. They are probably due to a weakening of the circulation in the liver or to interference with the metabolism of the cells as a result of the injection of foreign substances. Mechanical factors (pressure on the liver cells) may have an additional effect. This necrosis may be compared etiologically to the acute gastric ulcers which can be produced through a great variety of toxic substances in the guinea pig.

THE EFFECT OF THE INTRAVENOUS INJECTION OF SUBSTANCES AFFECTING TUMOR GROWTH ON THE CYCLIC CHANGES IN THE OVARIES AND ON PLACENTOMATA.*

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In preceding communications[1] we described the effect of the intravenous injection of various substances on tumor growth and on the liver of guinea pigs. In the present report we shall compare with the data obtained in these studies, the effect of the intravenous injection of some of these substances on experimentally produced placentomata, which represent rapidly growing tumor-like formations, and on the cyclic changes of the ovaries.

THE EFFECT OF THE INTRAVENOUS INJECTION OF HIRUDIN ON THE OVARIES.

In four experiments hirudin was injected intravenously[2] into female guinea pigs on the ninth or tenth day of the sexual cycle, and the animals died within a short time of the injection (four and one half to seven and one half hours after the injection; in one case the exact time of death was unknown, but the animal died within the first twenty-four hours). Six to seven days after ovulation, incisions had been made, under ether anesthesia, in the uterus. In these cases no marked changes were found in the ovaries, although, as we shall see later, a decided effect had been produced on the placentomata. There was perhaps a slightly increased degeneration of the large follicles, but it was not significant. There was in some cases an increase in the number of polynuclear leucocytes in the corpora

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[1] Fleisher, M. S., and Loeb, L., *Jour. Exper. Med.*, 1914, xx, 169. Loeb L., and Fleisher, M. S., *Jour. Am. Med. Assn.*, 1913, lx, 1857.

[2] 4 to 8 c.c. of a 0.25 per cent. solution of hirudin were injected each time.

lutea, mainly in the capillaries, but in some cases the leucocytes had entered the lutein cells.

In two cases the ovaries were examined one day after the injection of hirudin. In one of the cases the large and medium sized follicles and some of the small follicles showed degeneration of the granulosa. A mature follicle was, however, well preserved. In the corpus luteum there was some vacuolization in the periphery. In this case incisions had been made into the uterus six and one half days after ovulation, and three days later the hirudin was injected. In the other case hirudin was injected intravenously in a pregnant guinea pig. In this case we found also in all the large and medium follicles degeneration of the granulosa, while the mature follicles, as well as the small follicles, were well preserved. The capillaries of the corpora lutea were crowded with polynuclear leucocytes to such an extent that some of the capillaries appeared occluded; some lutein cells were necrotic. In a third case, in which without previous incisions into the uterus, hirudin had been injected six and one half days after copulation, the ovaries were normal.

We may conclude that within the first twenty-four hours after injection of hirudin the degeneration of the granulosa sets in at first in all the large and medium sized follicles and subsequently also in some small follicles. This degeneration of the granulosa is followed by ingrowth of connective tissue and complete atresia of the follicles. The mature follicles are considerably more resistant than the other large follicles,[3] and in a similar manner as they survive the injurious effect of those conditions which lead to ovulation, while the other follicles perish at that time, they may also survive the effect of hirudin, to which the other follicles succumb. We may find a large number of polynuclear leucocytes in the corpora lutea and also some degeneration of the lutein cells. At the same time we see that hirudin does not in all cases have this effect on the ovaries.

If about seven days after copulation incisions are made, under ether anesthesia, into the uterus, three days later hirudin is injected, and two or three days after the injection the ovaries are examined, we find only good follicles of small or small to medium size, while

[3] Loeb, L., *Centralbl. f. Physiol.*, 1911–12, xxv, 336; *Jour. Morphol.*, 1911, xxii, 37.

the large majority of follicles are found to be in various stages of connective tissue atresia. There may still be found some large follicles undergoing degeneration of the granulosa. In the corpora lutea we may or may not find collections of polynuclear leucocytes and some vacuolization and karyorrhexis in the lutein cells. In another case, however, in which nine days after copulation the hirudin had been injected without previous incision into the uterus, no changes were found in the ovaries.

In a further series of experiments two or three injections of hirudin were given in succession. In the majority of cases the incisions into the uterus were made six to seven days after copulation; three and five days following the operation hirudin was injected. The ovaries were examined from a few hours to three days after the last injection. In all these cases we find approximately the same picture in the ovaries; namely, good follicles of small or small to medium size and follicles in various stages of connective tissue atresia. There are especially many follicles in the last stage of connective tissue atresia; in one case some large follicles with degenerating granulosa were still present. In several other cases mature follicles were seen in which degeneration of the granulosa had set in. As we mentioned previously, mature follicles are very resistant, but they also degenerated under the influence of several injections of hirudin. In the corpora lutea we often found dilated vessels; polynuclear leucocytes might be present or absent. Some vacuolization of the lutein cells could sometimes be observed in the corpora lutea of these animals; it may, however, begin to set in normally at this period of the sexual cycle. The same changes were found in cases in which three instead of two injections of hirudin had been given.

In two cases of pregnancy where, without previous incisions into the uterus, two injections of hirudin were given, the changes were not as definite in the ovaries as in the previous experiments. In one of these two pregnant animals abortion followed the injection of hirudin.

From these experiments we may conclude that if six to seven days after copulation incisions are made into the uterus under ether anesthesia, and on the following days one or several injections of

hirudin are given, changes set in in the ovaries which correspond to those found at the time of ovulation; namely, a degeneration of all with the exception of the small follicles. These degenerative changes are followed by the same developmental changes which one of us has described previously in the ovaries.[4] In the cases that were observed for a certain period of time following the injection, the ovaries gradually resumed their normal function and ovulation occurred again. The corpora lutea are also sometimes, but not always, affected as a result of these injections. We found thus a second method of altering experimentally the periodicity of the sexual cycle; the first one consisted in the early extirpation of the corpora lutea, which one of us has described previously.[5] In several cases in which the injection of hirudin was given without previous incisions into the uterus, the ovaries were not at all, or were only slightly affected.

THE EFFECT OF THE INTRAVENOUS INJECTION OF HIRUDIN ON THE PLACENTOMATA.

In the majority of the guinea pigs referred to in the previous paragraph incisions had been made into the uterus of the anesthetized animals approximately five and one half to seven days after copulation. This operation was followed within the next week by one or several intravenous injections of hirudin. As a result of the incisions into the uterine wall placentomata began to develop within a few days after the operation. We were, therefore, in these experiments, in a position to determine the effect of hirudin on the placentomata.

In four animals in which the hirudin was injected about three days after the operation and in which the placentomata were examined, in three cases four and one half to seven hours, and in one case within the next twenty-four hours following the injection, as a result of the injection of hirudin by far the greater part of the developing placentomata had been destroyed by hemorrhage; however, small placentomatous areas near the mucosa had been pre-

[4] Loeb, L., *Jour. Morphol., loc. cit.; Virchows Arch. f. path. Anat.*, 1911, ccvi. 278.

[5] Loeb, L., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 17.

served; in the preserved areas there may have been some hyperemia; the number of mitoses in the cells of the placentomata was either very small or mitoses were entirely missing. The adjoining mucosa of the uterus was occasionally found to be edematous.

In two animals in which the placentomata were examined one day after the injection of hirudin, necrosis had set in in the greater part of the placentomatous tissue as a result of interference with the nourishment following the hemorrhages. In the majority of animals the placentomata were examined at a later period, about six to seven days after the operation, after one or several injections of hirudin had been given and several days after the last injection of hirudin. In these cases we noticed on macroscopical examination that the placentomata were much reduced in size; in some cases it was difficult to decide whether placentomatous tissue developed at all. Microscopically we find most of the placentomatous tissue destroyed by hemorrhage and necrotic. There were usually present some small islands of living placentomatous tissue which were situated near the mucosa, at places where the circulation had been less interfered with. But even in these areas, as well as in the adjoining mucosa, there were present indications of passive congestion. In the living placentomatous tissue mitoses were either missing or very few in number. The unfavorable circulatory conditions prevented probably a very active cell proliferation under those conditions. Hemorrhages could occasionally also be seen in the adjoining mucosa. In some cases collections of polynuclear leucocytes were present in the necrotic areas. They were in part attracted by the substances given off by the necrotic tissue. It is, however, probable that at least occasionally bacterial infection played a part. We found in some cases in which we examined the animals after injection of hirudin that certain bacteria developed at the place of injection; and even in the heart blood and in necrotic areas of the liver produced through these injections, bacteria could be discovered after intravenous administration of hirudin, while after injection of colloidal copper bacteria could not be found under those conditions. It is therefore possible that bacteria were retained in the necrotic area of the placentomata, and formed an additional attraction for the leucocytes. We observed one case in which hirudin failed to exert its usual effect on the placentomata.

In four cases the hirudin was once or twice injected at a certain stage after copulation without preceding incisions into the uterus. In two of these cases, early cases of pregnancy about ten to thirteen days after copulation, no abnormality could be found in the pregnant uteri; in two other cases in which the pregnancies were somewhat further advanced, hemorrhage took place in the neighborhood of the placenta, as a result of the injections of hirudin, with the subsequent death of the embryo in one case. We may assume that in both cases abortion would have taken place as a result of the injection, if the guinea pigs had been permitted to live for a longer period after injection of hirudin.

We may then conclude that intravenous injections of hirudin destroy in the majority of cases the greater part of the placentomata through hemorrhage, and they prevent the remaining placentomatous areas from active proliferation, probably also as a result of interference with the circulation. They may cause abortion in pregnant animals. While in the case of the placentomata hemorrhages are mainly responsible for the destructive action of hirudin, the action of hirudin on the ovarian cycle must have a different cause. Hemorrhages are missing in the ovaries. It appears probable that interference with the nourishment of the follicles as a result of the action of hirudin on the blood, or on the exchange of substances between blood vessels and tissue cells, is responsible for this effect.

THE RELATION BETWEEN INCOAGULABILITY OF THE BLOOD AND A TENDENCY TO HEMORRHAGE.

Our observations on the effect of hirudin on the placentomata suggest some general considerations on the possible relation between a tendency to hemorrhage and a decrease in the coagulability of the blood. In cholemia, in hemophilia, and in purpura such a connection exists. Morawitz and Bierich[6] found that if the blood of a dog is made free of fibrinogen and the blood coagulation is retarded, a hemorrhagic diathesis is not observed. These authors come therefore to the conclusion that there exists no direct connection between

[6] Morawitz, P., and Bierich, R., *Arch. f. exper. Path. u. Pharmakol.*, 1907, lvi, 115.

the retardation of the coagulation of the blood and the tendency to hemorrhage.

In our observation we find another instance of a parallelism in the retardation of the coagulation of the blood and in the tendency to cause hemorrhages in the case of hirudin. Intravenous injection of hirudin almost invariably causes hemorrhages into the placentomata. It frequently causes hemorrhages in mouse tumors after injection into the vein of the tail of the mouse. We stated in a previous paper[7] that hirudin frequently leads to necrosis in the liver of guinea pigs, and that in the neighborhood of this necrosis, hemorrhages may be found. We did not make a systematic study of other changes produced in the body through intravenous injections of hirudin, but we noticed in several cases erosions or ulcers and some hemorrhages in the wall of the stomach of guinea pigs, and in another case we found hemorrhage in the kidney tissue, blood casts and hyaline casts in the tubules, and some hyperemia of the glomeruli of the kidney. We may therefore conclude that injection of hirudin produces a tendency to hemorrhage in various parts of the body, and that the hemorrhages are prone to occur especially at such places where the blood vessels are not well formed, or are less resistant, as in tumors and in placentomata, or in the neighborhood of necrotic areas; and that in the stomach hemorrhages may be followed by digestion of the tissue through the gastric juice. It appears probable that there is a connection between the action of hirudin on the blood (effect on the coagulability or on the viscosity) and the tendency to hemorrhage which we noticed.

THE EFFECT OF VARIOUS OTHER SUBSTANCES ON THE OVARIES AND ON PLACENTOMATA.

A number of control experiments were carried out. We found changes in the ovaries after injection of hirudin especially in animals in which placentomata had been produced and in which hemorrhages into the placentomata had taken place. It was therefore conceivable that loss of blood in itself might produce some changes in the ovaries.

In each of three guinea pigs five cubic centimeters of blood were

[7] Fleisher and Loeb, *loc. cit.*

withdrawn from the jugular vein twice, an interval of two days elapsing between the first and second bleeding. On examination three days after the last bleeding, the ovaries and uterus were found to be normal in two animals; in the third animal the ovaries were hypotypical.[8] There were small and small to medium sized good follicles, many follicles in early and medium connective tissue atresia, there were many contracted follicles, and yellow scar-like bodies, the remains of former corpora lutea. The uterus showed low cuboidal epithelium in surface and glands; there were a few mitoses in the surface epithelium. The connective tissue of the mucosa was fibrillar and contained small nuclei. Some small mononuclear cells migrated from the connective tissue into the surface epithelium. It is not probable that the bleeding is responsible for the hypotypical changes in the ovaries of one of these animals and we may conclude that withdrawal of blood is without marked effect on ovaries and uterus.

In four anesthetized guinea pigs in which incisions into the uterus had been made about seven days after copulation, ten cubic centimeters of distilled water were injected intravenously two to nine days later. The examination was made one to four days following the injection. In three cases ovaries and placentomata were normal; in one case in which the animal was not in good condition one day after the injection, degeneration of the granulosa occurred perhaps somewhat earlier than usual, in some follicles of medium size. In a rabbit in which also placentomata had been produced experimentally, intravenous injection of distilled water had no influence on the placentomata. No effect was obtained through injection of distilled water in guinea pigs in which no incisions had previously been made into the uterus; one day after the injection the ovaries and uterus were found to be normal. We may conclude therefore that intravenous injection of distilled water has no distinct influence on the ovaries and uterus in the guinea pig.

In six guinea pigs the effect of repeated injections of colloidal solutions of copper on the ovaries and placentomata were tested. Six to seven days after copulation incisions into the uterus of the anes-

[8] Loeb, L., *Centralbl. f. Physiol., loc. cit.; Virchows Arch. f. path. Anat., loc. cit.*

thetized animals were made, and in four cases four, in one case three, and in the last case two injections of colloidal copper were given. Ten cubic centimeters of the solution were injected each time. The first injection was given three or four days after the uterine operation; the following injections were made on the following days, and the organs were usually taken out for examination one day after the last injection. The ovaries were found to be normal in all cases examined; in one case, however, all the large follicles showed degeneration of the granulosa. It is doubtful whether this was due to the influence of the colloidal copper. The placentomata in these cases were much larger than in the animals which had received hirudin; their size was approximately normal. There was, however, in most cases some hemorrhage and necrosis in the placentomata and it is possible that the injections of colloidal copper increased the hemorrhage and necrosis in the placentomata, which to some extent may be found even in guinea pigs that had not received any injection. There was, however, usually a considerable amount of living placentomatous tissue present with numerous mitoses. In two rabbits which had been similarly treated good deciduomata were found, and in one case a normal pregnancy was present.

We may conclude that repeated intravenous injections of colloidal copper are without effect on the ovaries of guinea pigs and without marked effects on the placentomata of guinea pigs and rabbits. They may possibly increase somewhat the amount of hemorrhage and necrosis, often to some extent normally present in further developed placentomata.

In twelve guinea pigs either one or two intravenous injections of nucleoproteid prepared from the kidney or lymph glands of cattle were given after incisions into the uterus of the anesthetized animals had been made about six to seven days after copulation. The first injection was given two to five days (in one case nine) after the operation; the second injection one to two days after the first one; two cubic centimeters of a 15 per cent. solution were given each time. Ovaries and uterus were in most cases taken out one to three days after the last injection. In the majority of cases the ovaries were found to be normal. In one case, however, one day after the injec-

tion there was much degeneration of the granulosa, even in medium sized and in some small follicles; in two other cases large follicles without degeneration of the granulosa were missing. It is therefore possible that in a few cases a slight effect on the ovaries was obtained; but in the majority of cases no effect was present. The placentomata were not noticeably influenced through the injection of nucleoproteid; occasionally some hemorrhages were observed in the placentomatous tissue.

In four guinea pigs in which six to seven days after copulation incisions into the uterus of the anesthetized animals had been made, nine to ten days after the operation intravenous injections of ten cubic centimeters of a 1⅔ per cent. solution of casein were given. The placentomata in three cases were taken out one day later; in one case the animal died ten minutes after the injection; in two cases there were extensive hemorrhages and necroses in the placentomata. In the two other cases the placentomata were in good condition. In two cases in which the ovaries were examined they were found to be normal. We have to consider the possibility that at this late stage of development of the placentomata (nine days after the operation) even mechanical factors (perhaps handling of the animals during intravenous injection) may be liable to cause some hemorrhage. In a rabbit injections of casein had no noticeable effect on the experimental placentomata or on pregnancy.

We may conclude that the various experimental interferences mentioned do not produce any marked or constant effect on the ovaries or on the placentomata.

SUMMARY.

If six to seven days after copulation incisions are made under ether anesthesia into the uterus, and on the day following this operation one or several injections of hirudin are given, changes set in in the ovaries which correspond to those found at the time of ovulation; namely, a degeneration of all the follicles with the exception of the small ones. These degenerative changes are followed by the same developmental changes as in the normal cycle. This represents a second method of altering experimentally the periodicity of the sexual cycle, the first consisting in the early extirpation of the corpora lutea described previously by one of us.

Under the same conditions intravenous injections of hirudin destroy in the large majority of cases the greater part of experimental placentomata through hemorrhages, and they prevent the remaining placentomatous areas from active proliferation, probably as a result of interference with the circulation. These injections may also cause abortion in pregnant animals.

Intravenous injections of hirudin produce a tendency to hemorrhage at various places in the body, and these hemorrhages are prone to occur, especially in rapidly growing tissues, where the blood vessels are less resistant, as in tumors and in placentomata, also in the neighborhood of necrotic areas. In the stomach the hemorrhages may be followed by digestion of the tissues through the gastric juice. It is probable that there is a connection between the action of hirudin on the blood (coagulability and viscosity) and the tendency to hemorrhage.

Withdrawal of blood, intravenous injections of distilled water, colloidal copper, nucleoproteid, or casein have no marked effect on the cyclic changes in the ovaries or on placentomata.

A STUDY OF THE CIRCULATION OF THE KIDNEYS FOLLOWING LIGATION OF ONE URETER.*

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In this work the perfusion box was the same as that used and described in a previous paper (1). Changes had to be made in the technique of perfusion, however, as in previous experiments only one kidney, the left, had been used.

In order to perfuse both kidneys simultaneously, two methods of procedure were tried. The first consisted in the use of one large aortic and two venous cannulæ; the second in using two arterial and two venous cannulæ. The first method, as far as it was tried, proved unsatisfactory because the serum could not be made to pass through normal kidneys at the expected rate. As the vessels given off by the aorta in this region other than the renal are very numerous, and as it was thought that the delay necessary to ligate these vessels brought about changes in the renal tissues, which probably interfered with the flow of ser m, the first method was ultimately discarded. The second metho. herefore was the one adopted. The cannulæ used in the second method were all large enough not to impede the flow. The diameters of the outlets of both arterial cannulæ were equal, the diameters of the inlets of both venous cannulæ were equal, and the diameters of the outlets of the cannulæ protruding from the side of the box were equal. The animal to be perfused was etherized and bled to death through the left femoral vessels. The abdomen was opened, and the intestines, stomach, and liver were removed. The portion of the animal between the diaphragm and the iliac crests was then taker ut, the aorta and vena cava were opened, and the cannulæ placed and ligated. The serum left the serum bottle through two glass tubes entering the arterial cannulæ under the same pressure.

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For several months the work was delayed because the serum could not be made to pass as rapidly through the right kidney as it did through the left. This discrepancy was thought to be due to technique rather than to a normal difference in the kidneys. Many possible sources of error were excluded until, finally, the discrepancy was overcome by the removal of the right adrenal.

In the rabbit the left adrenal lies almost directly over the aortic attachments of the renal arteries, and it had always been removed as it made more difficult the insertion of the cannulæ into these vessels. The right adrenal lies higher up and being less in the way had been left *in situ*.

As the removal of the right adrenal allowed the serum to pass more rapidly through the right kidney, it was thought that the removal of the left adrenal had been responsible for the ease with which the serum flowed through the left kidney. It was also thought, notwithstanding all proof to the contrary, that there might be some direct relation between the adrenals and the vasoconstrictor mechanism of the kidneys. In order to explain the above phenomena, the following experiments were performed.

RABBIT 7.

The Left Adrenal Was Left in Situ and the Right One Removed.

		Drops of serum.	
	Pressure.	Right.[1]	Left.[1]
After 5 min.	60 mm.	120 +[2]	120 +
After 10 min.	50 mm.	100	100
After 15 min.	50 mm.	81	84

In rabbit 7 the ligature around the left arterial cannula had been placed between the adrenal and the kidney. As the presence of the left adrenal did not seem to interfere with the rate of flow, it was thought that perhaps this ligature might have destroyed the relation between the adrenal and kidney on this side. The following experiments were therefore performed.

[1] The figures under the words "right" and "left" represent the number of drops of serum issuing in one half minute from the cannulæ protruding from the side of the perfusion box.

[2] It was found impossible to count accurately over 120 drops in 30 seconds. In such cases, therefore, where the serum issued from the cannulæ in distinct and separate drops, yet at a rate faster than 120 per 30 seconds, the sign 120 + was used.

RABBIT 19.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	40 mm.	95	120+
After 8 min.	40 mm.	100	120+
After 10 min.	40 mm.	90	120+
Right adrenal removed.			

RABBIT 21.

	Pressure.	Drops of serum. Right.	Left.
After 3 min.	40 mm.	85	120
After 5 min.	40 mm.	80	120
After 8 min.	40 mm.	80	120+
Right adrenal removed.			
After 10 min.	40 mm.	120	120+
After 15 min.	40 mm.	120+	120+

In these rabbits the ligatures around the arterial cannulæ were placed between the adrenals and aorta. During the first part of the experiment both adrenals were left *in situ*. During the latter part, the right adrenal was removed. In rabbit 19 air entered the right arterial cannula after the removal of the adrenal and the latter part of this experiment was consequently discarded.[3]

Having found a method whereby the serum could be made to flow simultaneously through both kidneys with the same pressure, at the same rate, two normal rabbits, each of which weighed between 1,500 and 2,000 grams, were perfused. The following results were obtained.

RABBIT 3.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	60 mm.	100	100
After 10 min.	50 mm.	103	100
After 15 min.	50 mm.	100	100

RABBIT 20.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	40 mm.	120	120
After 10 min.	40 mm.	120	120
After 15 min.	40 mm.	120	120

The flow through the normal kidneys in this series is apparently more rapid than that obtained previously (1), but this is explained by the small outlets to the cannulæ protruding from the side of the perfusion box, and the consequently smaller and more frequent drops.

In order to exclude nephropathic kidneys, only those rabbits were used the urine of which was free from albumen. Despite this, many of the rabbits on being opened were found to have diseased kidneys. In most of these rabbits the operation was continued and a ureter was tied as it was thought that the changes in nephropathic kidneys might be different from those in the normal kidney.

[3] Further attempts will be made to determine the exact relation between the adrenal gland and kidney perfusion.

In order to ligate the ureter the rabbits were etherized, the abdomen was opened, either the right or left ureter was ligated with silk, and the abdomen was then closed. Complete aseptic precautions were observed throughout. Several of the animals operated on died a few days after operation. The cause of death in these animals was not ascertained (2). It may be said, however, that the majority of the rabbits that died were from those that had changes in the kidney prior to ligation of the ureter.

With the object in view of finding out whether the intrapelvic pressure brought about by ligation of the ureter impeded circulation through the kidney (3) and whether this impediment gave rise to a compensatory increase in the flow through the other kidney, the first two rabbits operated on that were found to be normal were allowed to live three weeks before being perfused. Of these two animals, the hydronephrotic kidney of rabbit 1 was found to have become infected and consequently the changes produced therein by pressure could not be determined. The opposite kidney, however, other than being decidedly hypertrophied, showed no infection. Therefore, the animal was perfused with an idea of determining whether the flow through the hypertrophied kidney had materially increased. Rabbit 3 showed no infection.

RABBIT 1.

Right Ureter Ligated.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	40 mm.	5	Stream.[4]
After 10 min.	40 mm.	5	Stream.

RABBIT 3.

Left Ureter Ligated.

	Pressure.	Right.	Drops of serum. Left.
After 3 min.	40 mm.	Stream	0
After 5 min.	120 mm.	Pressure shut off	60
After 7 min.	60 mm.	Stream	14
After 10 min.	60 mm.	Shut off	16
After 14 min.	60 mm.	Shut off	14
	Sac punctured.		
After 14 min.	60 mm.	Stream	40
After 20 min.	60 mm.	Stream	48
After 25 min.	60 mm.	Stream	48

The conclusions drawn from the above experiments are as follows: (1) The intrapelvic pressure produced by ligation of a ureter impedes circulation through the kidney. (2) At the end of three

[4] Serum drops of approximately over 175 per minute lost their identity and merging formed a stream. In such cases the word "stream" is used.

weeks there is a compensatory increase in the rate of flow through the vessels of the opposite kidney. (3) At the end of three weeks after ligation of the ureter, changes occur in the kidney tissue which in themselves are sufficient to impede circulation.

On arriving at the above conclusions, the following experiments were performed with the object in view of determining how soon after ligation of the ureter the changes in the kidney tissue became sufficiently developed to produce an impediment to the flow. In this series the perfusion of the rabbits was begun as heretofore. When the flow through both kidneys had reached its maximum rate, the hydronephrotic sac was punctured and the perfusion then continued until the flow through the damaged kidney had again reached its maximum.

RABBIT 15.

Perfused Twenty-Four Hours after Ligation of Left Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	60 mm.	120+	9
After 8 min.	60 mm.	120+	9
	Sac punctured.		
After 10 min.	60 mm.	120+	10
After 15 min.	60 mm.	120+	10

For lack of serum the experiment was here discontinued.

RABBIT 12.

Perfused Forty-Eight Hours after Ligation of Left Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	60 mm.	120+	2
After 10 min.	60 mm.	120+	2
	Sac punctured.		
After 13 min.	60 mm.	120+	9
After 18 min.	60 mm.	120+	78
After 20 min.	60 mm.	120+	112
After 25 min.	60 mm.	120+	120+

RABBIT 13.

Perfused Forty-Eight Hours after Ligation of Right Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	40 mm.	9	120+
	Sac punctured.		
After 10 min.	50 mm.	90	120+
After 15 min.	40 mm.	120+	120+

RABBIT 14.

Perfused Seventy-Two Hours after Ligation of Left Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 3 min.	60 mm.	Stream	13
After 5 min.	40 mm.	Stream	10
After 8 min.	60 mm.	Stream	28
After 10 min.	60 mm.	Stream	42
After 12 min.	40 mm.	Stream	70
After 15 min.	40 mm.	Stream	70
	Sac punctured.		
After 20 min.	40 mm.	Stream	120

For lack of serum the experiment was here discontinued.

RABBIT 17.

Perfused Ninety-Six Hours after Ligation of Right Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 2 min.	60 mm.	70	120+
After 5 min.	60 mm.	120	50
After 10 min.	50 mm.	120+	50
	Sac punctured.		
After 12 min.	50 mm.	120+	80
After 15 min.	60 mm.	120+	120

RABBIT 18.

Perfused Ninety-Six Hours after Ligation of Left Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	60 mm.	120	40
After 8 min.	40 mm.	Stream	50
After 10 min.	60 mm.	Stream	50
	Sac punctured.		
After 14 min.	60 mm.	Stream	60
After 16 min.	60 mm.	Stream	55
After 20 min.	60 mm.	Stream	50

RABBIT 16.

Perfused One Week after Ligation of Right Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 2 min.	60 mm.	21	Stream.
After 5 min.	60 mm.	15	Stream.
After 6 min.	40+mm.	6	120+
	Sac punctured.		
After 10 min.	60 mm.	26	Stream.
After 12 min.	60 mm.	30	Stream.
After 14 min.	80 mm.	80	Shut off.
After 20 min.	80 mm.	82	Shut off.
After 22 min.	60 mm.	60	Shut off.
After 25 min.	60 mm.	60	Shut off.

Summary.—Increased intrapelvic pressure impedes the flow of serum through the kidney. Following ligation of the ureter a change occurs in the kidney tissues which offers an impediment to circulation other than that produced by pressure alone; this change

occurs one week after ligation. A compensatory increase in the rate of flow through the control kidney is first noticed seventy-two hours after ligation and is quite apparent at the end of one week.

HISTOLOGY.

The histological changes following ligation of the ureter have been described by many investigators (4, 5, 6). The changes will be described here in as far as they seem to be related to the problem in hand.

RABBIT 15.

Perfused twenty-four hours after operation.

Control Kidney.—No change is seen other than a slight edema associated with perfusion. A few of the glomeruli are distended with blood showing that the perfusion had not extended equally over all parts of the kidney.

Kidney with Ligated Ureter.—This kidney shows dilatation of all the convoluted and particularly the large collecting tubules near the end of the pyramids. Many of the tubules of Henle particularly the descending loops contain definite casts both hyaline and granular. Some are also found in the collecting tubules. In the cortex the primary convoluted tubules show slight swelling. Very considerable changes consisting in exudation and cell desquamation are found in the secondary tubules. None of these lesions are equally distributed; although there is general degeneration of the epithelium, the changes are more marked in single groups of tubules. The glomeruli are intact. The perfusion in this kidney is not complete, as many of the straight vessels in the pyramids contain blood. There is also much blood in the capillaries of the cortex.

RABBIT 13.

Perfused forty-eight hours after operation.

Control Kidney.—In this kidney the epithelium of the tubules in the pyramids is normal; the tubules are not dilated. There is an occasional cast in the Henle loops, and considerable edema. There is considerable edema in the tubules of the cortex with granular material in the lumen. Around all the glomeruli there is a crescent of coagulated exudation which consists in a granular mass enclosing large hyaline droplets. The perfusion here extended to all the glomeruli, the kidney being completely bloodless. This kidney is not absolutely normal, there being a few foci of cell infiltration and connective tissue increase in the cortex. The granular material in the convoluted tubules here is the same as that in the capsular spaces. The globules are usually of the same size and show a completely hyaline mass within a definite circle.

RABBIT 12.

Perfused forty-eight hours after operation.

Control Kidney.—This shows material of the same character in the capsular space, but very much smaller in amount and the hyaline globules are not so

marked. It is difficult in these cases to decide whether the material in the capsular space is due to perfusion and represents material which during the perfusion has passed through the glomeruli, or whether it had occurred during life. In many cases it is so abundant that the glomerulus is apparently compressed by it. The fact that it is not a condition common to a perfusion would seem to be evidence that it occurred during life, although it is perfectly possible that in these kidneys the glomeruli may have become more permeable.

Kidney with Ligated Ureter.—There is some dilatation of the collecting tubules, as in the previous case. Casts are present, but they are not so numerous in the tubules of the pyramids. The epithelium of the pelvic tubules shows a greater degree of degeneration, and the dilatation involves the smaller tubules of the pyramids relatively more than the larger. The dilatation involves also the tubules of the cortex, and in places even the primary collecting tubules. The secondary tubules are often dilated to double or more of the normal lumen. The epithelial degeneration in the primary convoluted tubules is well marked, and the tubules contain granular material derived from the epithelium. The most striking thing in this kidney is the greater extension of the dilatation into the tubules of the cortex.

RABBIT 14.

Perfused seventy-two hours after operation.

Control Kidney.—In this case the glomeruli are large and there is a varying amount of the same material in the capsule. Some contain a great deal, others but a small amount. In this kidney many of the glomeruli contain blood and have escaped the perfusion; elsewhere, there is much the same condition as in the kidney previously examined.

Kidney with Ligated Ureter.—The usual condition of dilatation of ducts and degeneration is seen. There is very little difference from that seen in the former kidneys.

RABBIT 16.

Perfused one week after operation.

Control Kidney.—In this kidney the exudate in the capsules is present. The epithelium generally is large and granular. There is some edema.

Kidney with Ligated Ureter.—There is the usual tubular dilatation. In the pyramids there is a distinct increase in connective tissue around the small blood vessels. There is throughout the kidney in both pyramids and cortex an increase in connective tissue. The glomeruli contain no exudate, but show an increase in the vascular cells which varies in degree.

RABBIT 3.

Perfused twenty days after operation.

Control Kidney.—The glomeruli on being measured are generally found to be larger than normally. Around most of them there is some granular material. The convoluted tubules show large, well granulated cells, and here and there in the pyramids there are foci of cellular infiltration.

Kidney with Ligated Ureter.—The dilatation of tubules here is but slightly apparent. There is a very marked increase of tissue with masses of cell infiltration in the pyramids, apparently extending from this into the cortex. The

increase of tissue in the cortex is diffuse, but accentuated in foci, and the dilatation of tubules is relatively more marked in the small collecting tubules of the cortex than elsewhere. There is marked thickening and proliferation of the epithelium of the cortex.

SUMMARY.

During the first four or five days after ligation of the ureter, the changes in the kidney are mainly degenerative in type. One week after ligation of the ureter connective tissue cells begin to appear infiltrating the kidney tissues. There is also an increase in the vascular cells of the glomeruli. These changes are well marked at the end of twenty days from the time of ligation. At the end of twenty days from the time of ligation there is also a marked hypertrophy of the control kidney, the glomeruli showing an average increase in size of twenty-five microns.

CONCLUSIONS.

During the first week following ligation of one ureter there is found an impediment to circulation which is removed upon opening the distended pelvis and reducing the intrapelvic pressure. This impediment, therefore, is probably due to the compression of the blood vessels within the kidney brought about by dilated tubules and unyielding capsule, and also possibly to disturbance of circulation produced by the distension of the pelvis. The venous and arterial branches passing over this may be either compressed or in various ways distorted. During this period the anemia produced by the impediment together with pressure and other factors gives rise to changes in kidney tissues which reveal themselves microscopically as a swelling and degeneration of the epithelial cells. Cloudy swelling was found by Karsner and Austin (7) two hours after experimental bland embolism of the renal artery.

At the end of one week following ligation of one ureter there is an impediment to the circulation other than that due to intrapelvic pressure. This impediment is probably due to cicatricial changes in the organ, which tend to maintain the tubules dilated and in a position of encroachment on the space normally allotted to blood vessels, and which manifest themselves microscopically as a connective tissue cell infiltration into the interstitial tissues of the

kidney, and an increase in the vascular cells of the tufts diminishing the caliber of the tuft capillaries.

The increased amount of work thrown upon the control kidney produces no lesions other than a hypertrophy, which permits a greater and more rapid circulation through its vascular system.

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BIBLIOGRAPHY.

1. Ghoreyeb, A. A., A Study of the Mechanical Obstruction to the Circulation of the Kidney Produced by Experimental Acute Toxic Nephropathy, *Jour. Exper. Med.*, 1913, xviii, 29.
2. Amos, B. S., On the Effect of Ligature of One Ureter, *Jour. Path. and Bacteriol.*, 1905, x, 265.
3. Lindemann, W., Ueber Veränderungen der Vascularisation der Niere bei Harnleiterunterbindung, *Centralbl. f. allg. Path. u. path. Anat.*, 1895, vi, 184.
4. Pearce, R. M., An Experimental Study of Nephrotoxins, *Univ. Penn. Med. Bull.*, 1903–4, xvi, 217.
5. Néfédieff, N., Sérum néphrotoxique, *Ann. de l'Inst. Pasteur*, 1901, xv, 17.
6. Castaigne, J., and Rathery, F., Lésions expérimentales de l'épithélium des tubes contournés, *Compt. rend. Soc. de biol.*, 1902, liv, 565.
7. Karsner, H. T., and Austin, J. H., Studies in Infarction. Experimental Bland Infarction of the Kidney and Spleen, *Jour. Am. Med. Assn.*, 1911, lvii, 951.